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CARBOHYDRATE METABOLISM AND THE EFFECT OF DECAPITATION AND DECEREBRATION UNDER NITROUS OXIDE ANÆSTHESIA.

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(Received September 5, 1933.)

In continuation of the investigations by Evans, Tsai and Young [1931*a*, *b*] on the carbohydrate metabolism of cats decapitated or decerebrated under ether, and of those of Murphy and Young [1932] on the influence of a variety of other anæsthetics on carbohydrate metabolism, we have studied the action of nitrous oxide anæsthesia. We did so because some experiments with this anæsthetic by Peterson [1933] seemed to indicate that it might have every advantage over ether. Though our results only partially bore out this hope, they showed that, among serious drawbacks, some decided advantages were to be had by its use, and that it is an anæsthetic very easily given to cats, and likely to be of at least as much use in physiological work as in the clinic.

Nitrous oxide does not produce its effects solely by causing oxygen lack, as was shown by Paul Bert [1878, 1879, 1888], though Wieland [1922] does not regard it as a narcotic in the ordinary sense of the term. That it does not combine with hæmoglobin was shown by Hermann [1864] and again by Siebeck [1909], who also showed that the gas is more soluble in blood than in water. Since there is always definite cyanosis under nitrous oxide anæsthesia we had feared that there would be a large formation of lactic acid and rise of blood sugar, and the investigations of its effects on the *p*H and alkali reserve of the blood by Cullen *et al.* [1923] and by Leake and Hertzman [1924], in so far as they were not explainable as due to over-breathing, also pointed in that direction.

Methods.

Three methods have been tried for administering the gas, viz. the bag and mask for induction, and continuous insufflation, or a closed circuit for prolonged maintenance. Whichever method was used, the mixture of gas and oxygen was always made after passing

[1] Holder of Royal Society of Canada Fellowship in Biology.

the gas from cylinders through simple bubbling flow-meters, of a type sometimes used for clinical purposes, each consisting of a wash bottle with the brass down-tube perforated with a row of fine holes: an ether bottle was also added for occasional use (Fig. 1).

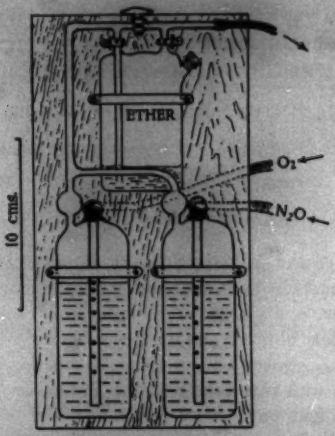

Fig. 1. The bubble meter for administration of N_2O, oxygen or ether.

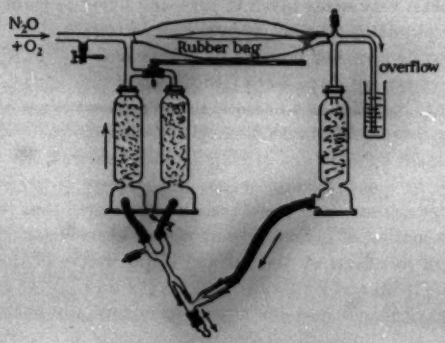

Fig. 2. The closed circuit apparatus.

In the experiments in which the animals were allowed to recover, the inhaler used for induction was used throughout. This consisted of an airtight silk bag (air cushion) of about 2 litres capacity: at one corner was an inlet tube with tap, and at the opposite one a brass

funnel with tube about 3 cm. wide and 3 cm. long; over the wide part of the funnel a piece of motor inner tube about 10 cm. long was fixed so as to form a sleeve into which the animal's head could be thrust.

For experiments involving prolonged anæsthesia, or operations on the neck, continuous insufflation may be used. This we carried out, after induction, by passing into the trachea, through a small incision, a fine gum-elastic catheter, through which a sufficiently rapid stream of gas was passed. This method though satisfactory is extravagant, and we only used it once, since we found the closed circuit arrangement better.

This is shown in Fig. 2. After induction with the bag, a short wide tube was tied into the trachea, as low down as convenient, and connected with the apparatus, previously filled with gas and oxygen. The expired air passed through a valve to one of the two soda-lime towers and into the rubber bag, thence into a moistening tower filled with wet cheese-cloth, and so to the inspiratory valve. A fresh supply of gas and oxygen was continuously passed from the bubble meters into the bag, at such a rate that there was an escape at the overflow valve (a tube dipping into water) during expiration.

For decapitation two methods were tried. In the first, the animal was anæsthetized with the use of the bag, and tracheotomized. Instead of the closed circuit arrangement a small mobile bag (small football bladder), filled from the meters, was connected: it was furnished with a T-tube by which the bag could be emptied and refilled at intervals. Manipulation of the head was thus unimpeded. The second method necessitated giving the gas for only a few minutes; as soon as anæsthesia was fully established by the bag, the spinal cord was severed, and the brain destroyed by means of a sharp bradawl passed through the atlanto-occipital joint. The anæsthetic was withdrawn, tracheotomy quickly done and artificial respiration started. The head was then removed in the usual manner, decapitation usually being complete in from 5 to 8 min. from the commencement of induction.

It was observed that anæsthesia was more easily maintained after both carotids had been tied, and for this reason we found decerebration was easy without the use of any other inhalation device than the bag. We performed the operation with the opened skull, scooping out the brain and lightly packing the cavity with wool. Section was at the level of the anterior colliculi above, and between 2nd and 3rd cranial nerves below. The pituitary fossa was intact. Time from 4 to 10 min. from start of induction. Decerebrate rigidity set in almost instantly and the preparations were in very good condition. Blood-pressures, recorded at intervals, showed good maintenance for decerebrated animals, but rapid decline after decapitation, particularly when effected by the bradawl.

The blood was sampled from the ear before anæsthesia, and, in the case of the animals for recovery, at intervals throughout the experiment. In other cases the blood was taken from the carotid artery. The sampling of the liver, and determinations of glycogen, blood sugar and lactate were performed as described in the paper by Evans, Murphy and Young [1932].

Analysis of the gas mixtures was sometimes made by the method described by Krogh and Lindhard [1912] and Boothby and Sandiford [1915]: the technical difficulties of the analyses are considerable. Since the nitrogen content was small, was obtained by difference, and in any case represented the results of analysis of only about 2·5 c.c. of gas, it bears the chief error of the analysis. Oxygen and CO_2 results, being made on 10 c.c. samples, are more accurate. It is unfortunate that ordinary methods for determining oxygen saturation of the blood are vitiated by its large content of nitrous oxide, and we could think of no simple and reliable way of circumventing this difficulty. Hence we could rely only upon the colour of the blood as a guide to its oxygenation.

Results.

Course of anæsthesia.

Induction proceeds more quietly if from 5 to 10 p.c. of oxygen is added to the nitrous oxide, as the latter when pure appears to cause asphyxial sensations with consequent struggling. Unconsciousness is usually produced with no struggling, and in from 1 to 2 min., after which the oxygen content can be slightly increased, particularly if one or both carotids have been tied, and if prolonged anæsthetization is required. Samples drawn from the interior of the bag in one experiment, at intervals from 5 to 40 min. from commencement, showed oxygen contents from 7·3 to 15·5 p.c., carbon dioxide from 0·18 to 1·57 p.c., and N_2O from 84·39 to 92·13 p.c., according to the oxygen regulation and to whether the sample was taken just before or just after the contents of the bag had been replenished. Given by this closed method there is necessarily rebreathing and a considerable CO_2 content just prior to replenishing the bag, which was done by pressing out its contents about once a minute and allowing it to refill.

By the closed circuit method extensive rebreathing can, if desired, be obviated. Table I shows the composition of the gases on the inspiratory

Table I.

Exp. 339.		CO_2 p.c.	O_2 p.c.	N_2O p.c.	N_2 p.c.
About 20 min. after induction.	Inspiratory	0·15	16·1	83·25	0·5
	Expiratory	2·84	12·9	83·8	0·46
About 40 min. after induction.	Inspiratory	0·10	15·3	81·0	3·6
	Expiratory	2·63	11·8	81·8	3·77
About 75 min. after induction.	Inspiratory	0·12	20·3	74·9	4·68
Exp. 340.					
About 40 min. after induction.	Inspiratory	0·2	7·6	92·2	0·0
	Expiratory	1·48	7·55	90·27	0·70
About 75 min. after induction.	Inspiratory	0·29	12·03	86·3	1·38
	Expiratory	0·48	12·25	86·8	0·47

and expiratory sides of the apparatus at intervals during two experiments. The expiratory and inspiratory samples were not taken simultaneously, and therefore the oxygen differences are not strictly comparable with the CO_2 differences.

It would seem from these results, which were made on cats with one carotid tied, that anæsthesia can be maintained even when there is as much as 15 p.c. of oxygen in the mixture. Although the arterial blood was somewhat darker in colour than might have been anticipated from

the oxygen content of the gas mixture, anoxæmic symptoms were not pronounced.

For recovery experiments we have usually given the mixture for ½–1 hour, but in terminal experiments as much as 7 hours has been given without noteworthy deterioration in the animal's condition.

The most obvious advantage of nitrous oxide anæsthesia, and one which for physiological purposes outweighs all others, is the rapidity and completeness of the subsequent recovery, which even in the least favourable example contrasts dramatically with recovery from the usual volatile anæsthetics. In from 20 to 40 sec. after removal of the mask signs of consciousness are evident, the animal raises its head and looks at its surroundings. In 2 min. it has completely recovered, sits up and almost invariably purrs. Only rarely is there over-breathing or struggling during or following recovery, or any other indications than those of normal or even enhanced happiness.

Effect of nitrous oxide anæsthesia on blood sugar and lactate.

Effect on blood sugar. This was first studied in animals which were anæsthetized from the bag for a time, no operation being done other than that involved in drawing samples of blood from the ear at intervals during the anæsthesia, and the subsequent period of recovery, in the same way as was done prior to induction. The results of these experiments, carried out on cats, some of which had fasted for about 16–20 hours, and others for 44 hours, were rather variable, particularly for the animals subjected to the longer fast. Results of a typical experiment are shown in Fig. 3, the dotted line in which shows the mean results for seven experiments. (In one of these the closed circuit was used with tracheotomy and ligature of the carotids.) The most usual result, as both these curves show, is that there is a rapid rise in blood sugar, though not generally to a higher level, and sometimes even less high, than that when ether is used. In four experiments out of the seven the blood sugar fell off again in from 5 to 30 min. after the induction; in one of these (the closed circuit) the blood sugar, initially 75 mg./100 c.c. rose in 30 min. to 128 mg./100 c.c. and then fell, reaching 93 mg./100 c.c. in 65 min. In the remaining experiments, after induction, there was a continual, though ultimately slower, rise in blood sugar throughout the period of anæsthesia. The highest level reached in any of these seven experiments was 271 mg./100 c.c. in 58 min. (initial 92 mg./100 c.c.).

It has often been supposed that the rise of blood sugar which accompanies the administration of a volatile anæsthetic, such as ether, is due to

accompanying oxygen lack. If this is so, it might have been expected, since the blood was nearly always very dark in colour and the animals sometimes restless, that in our experiments the rise would have been greater than it was actually found to be. Asphyxiation acts rapidly on the blood sugar. Thus, asphyxiation by clamping the trachea of a cat, under amytal, for 2 min., raised the blood sugar from 136 to 251 mg./100 c.c. 1 min. later, while under luminal the rise was from 89 to 210 mg./100 c.c. in 2 min. from onset. Yet Banerji and Reid [1933] have shown that amytal considerably reduces the hyperglycæmic response to adrenaline or

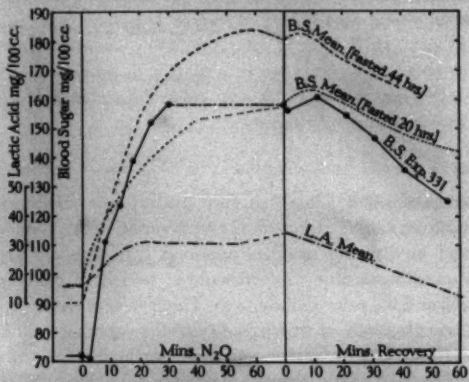

Fig. 3. Blood sugar and lactic acid for 1 hour, followed by recovery. Mean curves dotted. Exp. 331 as example.

ether. In spite of the dark colour of the blood in our experiments, we do not think the oxygen lack was the sole cause of the rise of blood sugar. In order to test this we have in some experiments transferred the anæsthesia

Exp. 327. Cat fasted 16 hours. Closed circuit.

Time (min.)	Procedure	Blood sugar mg./100 c.c.
−8	Sample from ear	111
0	$N_2O + O_2$ from bag	—
2–8	Tracheotomy, etc.	—
24	Carotid sample (dark)	257
69	,, ,, ,,	222
84	,, ,, ,,	229
104	,, ,, ,,	227
107	Transfer to ether and O_2	—
121	Sample (bright red)	235
179	,, ,, ,,	256
204	,, ,, ,,	256

without interruption to ether and oxygen; the result was that, in three out of four experiments, ether and oxygen, though it caused the blood to become bright scarlet in about 1 min., produced a further slight rise in blood sugar; in the fourth case there was a slight fall. The protocol of Exp. 327 illustrates the definite rise.

After recovery from anæsthesia there is often for several minutes a definite unexplained rise in blood sugar which shows on the curves of Fig. 3; this is not necessarily a continuation of an existing tendency to rise during the last minutes of the anæsthesia, since it is seen, as in the protocol of Exp. 329, in instances where the sugar content was falling at the end of the anæsthesia.

Exp. 329. Cat fasted 48 hours. All samples from ear. Anæsthesia by bag.

Time (min.)	Procedure	Blood sugar mg./100 c.c.
−4	Sample	82
0	$N_2O + O_2$	—
5	Sample	91
17	,,	96
28	,,	114
33	,,	108
34	Allowed to recover	—
39		118
54		101
65		98
94		81

The recovery rise was certainly not due to fright or uneasiness. The only suggestion we can make is that in nitrous oxide anæsthesia two opposed processes may be at work, one of which, common to the action of many anæsthetics, is a mobilization of blood sugar, the other one, seen with nitrous oxide and perhaps with other anæsthetics, and sometimes preponderating, is a reduction of mobilization.

We thought it possible that nitrous oxide, although it might, by inducing oxygen lack, lead to a secretion of adrenaline, might in some way hamper the liver from responding to this in the usual way. But we found that the subcutaneous administration of 2 mg. of adrenaline to a cat under nitrous oxide anæsthesia was followed by a further rise (of 40 mg./100 c.c.) to 314 mg./100 c.c. in blood sugar 2 hours later, and therefore abandoned the idea.

The animals which had fasted for only 16 hours gave rather more regular results; mean results for four experiments are shown in Fig. 3. The curve otherwise shows similar features to that for the animals which had fasted for 44 hours and calls for no detailed comment. It should be said, however, that, although our mean curve does not indicate it, an

animal which has fasted for the shorter time gives on anæsthetization a larger rise of blood sugar than the same animal when fasting longer. Owing to the differences between individual animals, this fact can only be revealed when the same animal is used for both experiments.

The experiments made with the use of the closed circuit apparatus gave similar results and need no separate description. Fig. 4 shows the mean results for animals fasted 20 and 48 hours respectively, and also the actual results of an experiment in which the animal was kept under gas and oxygen for about 5½ hours: the highest blood sugar reached during that time was 192 mg./100 c.c. at 168 min. The difference between long and short fasts is here shown on the mean curves (dotted lines).

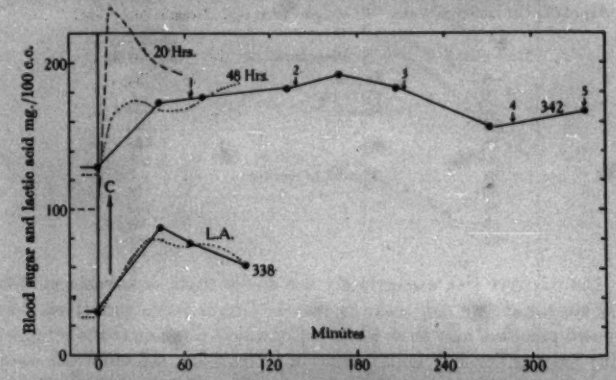

Fig. 4. Blood sugar and lactic acid in closed circuit experiments. Induction at 0, connection to circuit at about *C*. Mean curves for blood sugar of cats fasted for 48 hours (five experiments), and 20 hours (three experiments); and of lactic acid 16 to 44 hours (four experiments).

Exp. 342. Fasted 44 hours. At 1, 6 mg. ergotoxin given intravenously: at 2 and 4, vagi stimulated, right and left alternately, for 5 min. each (½ sec. tetani repeated 25 times per minute. Coil at 7, with 3-cell accumulator): at 3 and 5 stimulation of vagi stopped.

Exp. 338. Fasted 44 hours. Air of circuit at expiratory valve at 44 min. = 9·7 p.c. O_2 and 2·19 p.c. CO_2. Vagi cut and stimulated as mentioned above at 76 min. until end of experiment.

Effect on blood lactate. In a few minutes after induction is begun, the blood lactate rises (Figs. 3 and 4), and in the experiments with the closed circuit the rise is greater and more prolonged than in those carried out with the bag and followed by recovery, and in which the figure usually

reaches about 30–50 mg./100 c.c. No striking difference is seen between the animals with long and short fasting periods. Even in the closed circuit experiments the rise is usually not enormous, the average of the maxima being about 80 mg./100 c.c. Not infrequently there is a subsidence of lactate in the later stages of anæsthesia as shown by Exp. 338 (Fig. 4).

No doubt the oxygen lack, together perhaps with some direct effect of the nitrous oxide, acts on the central nervous system, and that the rise of lactate, like that of blood sugar, is due largely to a discharge of adrenaline into the circulation seems most probable.

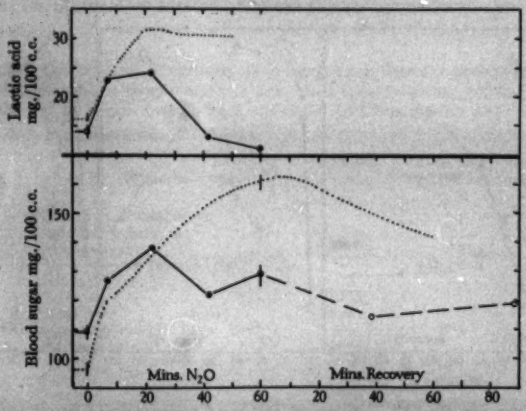

Fig. 5 (Exp. 354). Cat fasted 16 hours. Right splanchnic cut and left suprarenal removed 7 days previously. Blood sugar and lactic acid curves during anæsthetization with N_2O, and blood sugar during 89 min. after recovery. The dotted curves show, for comparison, average results for cats with suprarenals intact fasted 16 hours.

This is confirmed by previous removal of one suprarenal, and section of the contralateral splanchnic nerve. The rises of blood sugar and lactate resulting from anæsthetization with nitrous oxide and oxygen, though not abolished, are then much reduced (Fig. 5).

Some of the lactate, however, appears to be the result of oxygen lack, acting either on spinal centres, or, more probably, on the muscle direct. Thus, in one experiment 69 min. after decapitation, and when the blood sugar and lactate had fallen considerably, we gave, by artificial respiration, a mixture of nitrous oxide, and an hour later the blood sugar had

fallen from 169 to 147 mg./100 c.c., but the lactate had risen from 49 to 72 mg./100 c.c.

Pregnant animals. In several instances we used pregnant cats, and in these the effects of prolonged fasting are much more striking than in non-pregnant animals. A good example is shown in Fig. 6, *A*, *B* and *C*. In *A* and *B* the animal was near full term; for *A* a fast of 44 hours and for *B*, 2 days later, one of 16 hours was given; a few hours after the conclusion of that experiment the animal gave birth to a litter: 4 days after this the lactating animal, fasted for 44 hours, was again used, and gave the data

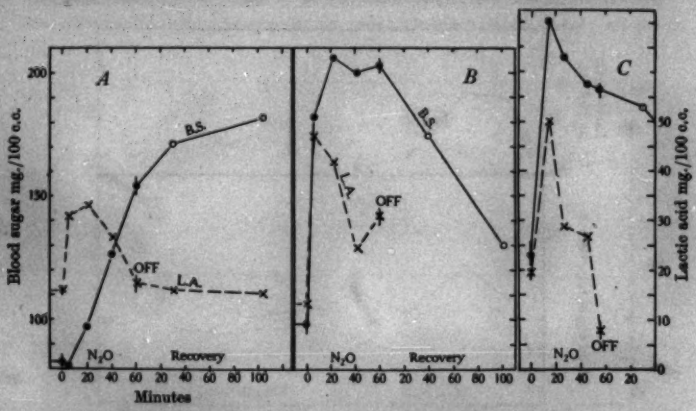

Fig. 6 (Exps. 348, 350, 352). The same cat, at *A* and *B* pregnant near full term, at *C* 4 days after delivery. Blood sugar and lactate curves during and after N_2O anæsthesia. N_2O given at 0, off about 60. *A* and *C*, fasted for 44 hours, *B* for 16 hours.

of *C*. It will be seen that in the pregnant animal the rise of blood sugar and of lactic acid were much more pronounced when only the shorter fast had been endured, and further that the type of recovery curve was different, for whereas in the long-fast experiment the rise of blood sugar continued long into recovery, the falling-off was rapid in the short-fasting one. The lactating and long-fasted animal resembled the pregnant animal with the shorter fast. In another case we obtained only 9 mg./100 c.c. rise on administering N_2O for 23 min. to a 48-hour fasting pregnant cat at term, though a normal rise was obtained a week after delivery. If the rise of blood sugar is to be accounted for as due to a secretion of adrenaline, then it is necessary to explain why the lactic acid

falls so promptly, even during anæsthesia. It seems not unreasonable to suppose that the phenomena are explicable on the ground that there is a large demand for carbohydrate in the body owing to the requirements of the fœtuses and/or of the lactating mammary glands. If we regard the amount of liver glycogen as an important factor in determining the extent to which the blood sugar will rise under anæsthetization, then it would seem that in the pregnant animal the glycogen store is reduced more rapidly by starvation than it is in the non-pregnant. We did, in two experiments, determine the liver glycogen of pregnant cats fasted for 48 hours and decapitated or decerebrated, and found it even lower than is usual for animals so treated. The results are given in the protocol of Exp. 344.

Exp. 344. Cat, full term. Preliminary blood sugar 90 mg./100 c.c. Gas and oxygen at 0; at 13 min. decapitation complete. At 18 min. blood lactate = 104 mg./100 c.c. Sugar = 97 mg./100 c.c. At 31 min. liver glycogen = 0·019 p.c.; at 228 min. 0·028 p.c.

Exp. 355. Six fœtuses 6·5 cm. in length.

Time (min.)	Remarks	Glycogen p.c.	Lactic acid mg./100 c.c.	Blood sugar mg./100 c.c.
−6		—	15·0	97
0	Nitrous oxide-oxygen on			
1	Under anæsthetic			
6	Decerebrated			
14	Blood-pressure 165 mm. Hg	—	113·0	161
26	Maternal liver	0·03		
36	Placenta	0·112		
	Fœtal liver	0·05		
87	Blood-pressure 116, temp. 39·1° C.	—	20·3	235
96	Maternal liver	0·036		
195			19·9	278
202	Maternal liver	0·03		
208	Placenta	0·072		
	Fœtal liver	0·036		

Liver residue 54 g., initial weight = 62 g.
Placenta residue 50 g., initial weight = 56 g.

It will be noted that the total amount of glycogen in the entire placental tissue, though not great (0·06 g.), was more than that in the maternal liver (0.02 g.), while the fœtal livers in all contained less than 2 mg. of glycogen between them.

Decapitation and decerebration under nitrous oxide.

Decapitation. Fig. 7, *A*, *B*, *C*, *D*, shows the changes in blood sugar and liver glycogen (*A* and *B*) and lactic acid (*C* and *D*) following decapitation by the two methods in animals fasted for 16 hours (*A* and *C*) or 44 hours (*B* and *D*). Exp. 344 was on a pregnant cat near term. It will be seen that decapitation by either method gave much greater increases in both sugar

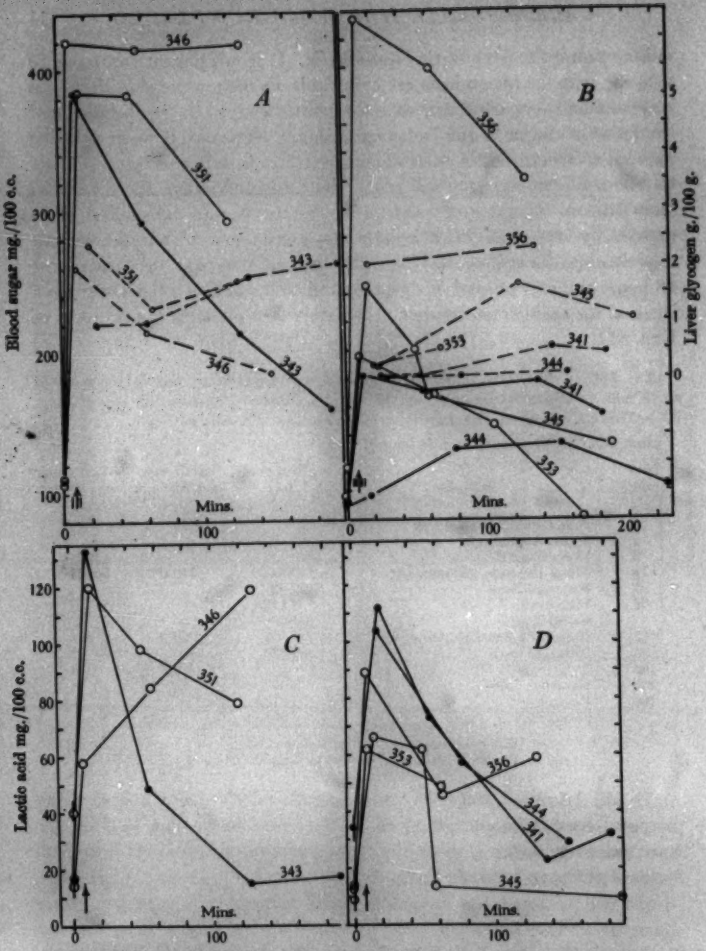

Fig. 7 (Exp. numbers as on curves). Decapitation under N_2O by bradawl (circles) or by Sherrington method (black dots). Curves *A* and *B* for blood sugar (lines) and liver glycogen (broken lines): curves *C* and *D* for blood lactate in the corresponding experiment. Induction at 0. Decapitation at arrow. *A* and *C*, fasted 16 hours; *B* and *D*, fasted 44 hours.

and lactic acid in the blood than result from the N_2O alone (cf. Fig. 3), but that usually these subsided later. The rises in blood sugar and in blood lactate on decapitation were greater after a 16-hour than after a 44-hour fast.

Liver glycogen showed a long-lasting fall in the three short-fasted cats, but considerable recovery occurred later in two of them, accompanied by a definite fall in blood sugar and lactate; the third one (Exp. 346) showed a continued fall in liver glycogen with a maintained blood sugar and a rising lactate throughout the experiment. It was found at autopsy that this animal was suffering from an old ulcer on the thoracic wall, and that the liver was abnormal, being much paler and firmer with a higher fat content than usual. All the cats fasted for 44 hours showed some subsequent recovery of liver glycogen, though only in one case (Exp. 345) was this considerable. Exp. 344 has already been referred to as illustrating the low level of liver glycogen towards the end of pregnancy. On the whole, the recoveries of liver glycogen were of about the same order as those found by Evans, Tsai and Young after decapitation under ether, in which in 150 min. there was on the average a 2·2-fold increase: in the present experiments the glycogen content, probably because of the combined effects of oxygen want and of intense response to section of the nervous system, was if anything lower just after decapitation than in the experiments by Evans, Tsai and Young. It will be shown later that probably on the average about 84 p.c. of the liver glycogen was lost when decapitation was done under nitrous oxide, as compared with about 75 p.c. when done under ether, so that N_2O seems to present no advantage over ether as an anæsthetic preliminary to decapitation. We had supposed that ether might have the drawback of being only slowly eliminated, and that in consequence the recovery of liver glycogen might from the first have been more rapid when N_2O was used, but our experiments do not bear out this supposition: such advantages as N_2O possesses seem to be at least balanced by the drawbacks that reflex hyperglycæmia is considerable because anæsthesia is light, and that oxygen lack is present at the time of decapitation.

Decerebration. The effects of decerebration on the blood sugar and lactate and on liver glycogen are similar to those described by Evans, Tsai and Young for decerebration under ether. This is illustrated by Fig. 8, *A* and *B*. In the animal used for *B* the left suprarenal was removed and the right splanchnic divided 16 days previously. In *A* the cat was fasted for 44 hours, and had 3 hours previously furnished the recovery curve shown in Fig. 6, *C*. Cat *B* was also fasted for 44 hours, nevertheless

the rise of blood sugar in *B* was much smaller and was followed by a fall, whereas in *A* the level remained high. If this high blood sugar was due to a constant liberation of adrenaline, then the lactic acid should also have remained high, unless, which is indeed quite possible, the liver was able to remove the lactic acid more rapidly than it was being formed in the muscles. In *B*, where we may suppose the initial high lactic acid to have

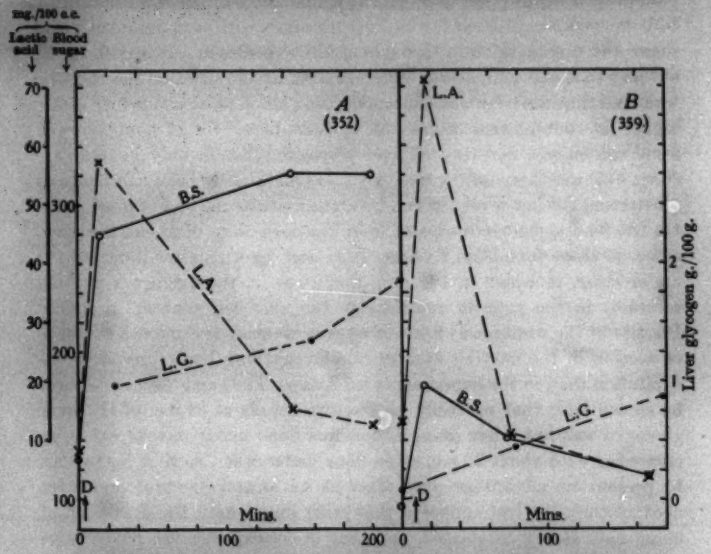

Fig. 8. N_2O at 0. Decerebration at *D*. Blood sugar, lactic acid and liver glycogen p.c. *A*, cat fasted 44 hours (lactating, Fig. 6 *C* made 3 hours previously); *B*, right splanchnic cut and left suprarenal removed 16 days before, fasted 44 hours.

chiefly resulted from powerful muscular contraction at decerebration, the fall was still more rapid. Glycogen recovery took place at about the same rate in both experiments.

Source of the regenerated liver glycogen.

The glycogen formed after decapitation or decerebration might arise from muscle glycogen *via* blood lactate [Cori, 1931], or from blood sugar, or be formed by glyconeogenesis; these possibilities were discussed by Evans, Tsai and Young [1931*b*], who, although lactic acid determina-

tions were not regularly made in all their experiments, thought it probable that glyconeogenesis must account, at all events in part, for the increases they found. It is of interest to review some of the present experiments, which in some respects are more complete, in order to see whether this conclusion could be held still to be valid, or whether, on the other hand, the increases of liver glycogen could be covered by the concurrent reductions in blood and tissue lactate and sugar.

It is probably justifiable to suppose that there is no continued secretion of adrenaline after decapitation, nor after decerebration with the suprarenals inactivated, so we may consider that in those experiments there has been no continued conversion of the muscle glycogen *via* lactate, into liver glycogen. Similarly, there is reason to believe that no muscle glycogen is laid down after decapitation. In the ordinary decerebrate animal it may be otherwise, and so there might in that case therefore be some carbohydrate available other than that as existent or glyconeogenetic liver glycogen or as free glucose. In making these calculations we have assumed that a 3 kg. cat after decapitation will utilize by combustion 1 g. of glucose per hour [cf. Evans, Tsai and Young, 1931 *b*, p. 96], and, further, that blood sugar and lactate, except for the first few minutes after decapitation or decerebration, are distributed throughout a mass of blood and tissue together equal to one-third of the body weight.

If, therefore, we determine the liver glycogen, blood lactate and blood sugar soon after decapitation, and again after a suitable interval of time, we have the data from which to determine whether the liver glycogen formed can all be accounted for by the observed reduction in blood sugar and lactate, or whether some carbohydrate must have arisen by gluconeogenesis, since we assume that

Glucose burned + liver glycogen formed
= loss of sugar and lactate
+ carbohydrate formed by gluconeogenesis.

Table II shows the results of such calculations.

There is, as shown in Table II, a single experiment, in which there is, fortuitously, an exact balance; the other five experiments all suggest that more carbohydrate has been available for the body than can be accounted for except as explained above, the amount varying from 0·6 to 1·22 g. per hour. The last figure, in accordance with our expectations, was obtained from a decerebrate cat and might therefore partly represent a conversion of muscle glycogen into available carbohydrate. In the other instances this explanation is unlikely, because even if conversion were quanti-

TABLE II. Calculation of gluconeogenesis.

Exp.	Time between samples min.	Wt. of liver when sampled g.	Glycogen in liver g.	*A* Gain in liver glycogen g.	*B* Total free sugar in body g.	*C* Total free lactic acid in body g.	*D* Joint reduction of tissue sugar and lactic acid g.	*E* Glucose lost by combustion (calc.) g.	*F* Sum of *A* and *E*	Excess of *F* over *D* = gluconeogenesis g.	Gluconeogenesis per hour g.
343	172	71	0·68	0·64	4·6	1·59	4·09	3·45	4·09	0·0	0·0
Decapitate, fast = 16 hours		64	1·32		1·9	0·20					
345	112	60	0·12	0·81	2·46	0·67	1·52	1·86	2·67	1·15	0·6
Decapitate, fast = 50 hours		56	0·93		1·50	0·11					
353	49	71	0·171	0·191	2·07	0·66	0·33	0·88	1·07	0·74	0·91
Decapitate, fast = 44 hours		69	0·362		1·86	0·54					
358	162	98·3	5·21	0·17	1·78	0·55	0·92	2·47	2·64	1·72	0·64
R.S.A.* Decapitate, fast = 16 hours		94·5	5·38		0·96	0·45					
359	147	91·0	0·06	0·70	2·10	0·68	1·28	3·10	3·80	2·52	1·03
R.S.A.† Decerebrate, fast = 24 hours		85·1	0·76		1·45	0·05					
352	196	80·0	0·77	0·47	3·07	0·63	0·04	3·55	4·02	3·98	1·22
Decerebrate, fast = 44 hours		67·0	1·24		3·52	1·14					

* R.S.A. = Right suprarenal removed and left splanchnic divided 9 days previously.
† R.S.A. = Right suprarenal removed and left splanchnic divided 16 days previously.

tative it would necessitate a fall of muscle glycogen of the order of 0·04–0·07 g./100 g. per hour, whereas it has been repeatedly shown that the muscle glycogen of the decapitate cat does not change so rapidly, if at all.

Calculation of the initial liver glycogen.

Important as it is to know the initial glycogen content of the liver, it is impossible to take early samples of liver from which this may be inferred, as was done for ether decapitations by Evans, Tsai and Young. But since we have no reason to suppose otherwise than that the liver glycogen lost during the procedures ending with decapitation enters the blood and inundates the tissues, the amount so lost may be calculated roughly from the pre-anæsthetic and post-operative blood sugar, and from the liver weight and liver glycogen content just after operation. We here assume once more that, except for the first few minutes after decapitation or decerebration, a mass of blood and tissues equal to one-third of the body weight is in equilibrium with the blood as regards glucose content. An example will make the calculations clear.

Exp. 345. Cat, weight 3·0 kg. Initial blood sugar 92 mg./100 c.c. Blood sugar 10 min. after decapitation 246 mg./100 c.c. Initial weight of liver 60 g. Glycogen content 10 min. after decapitation 0·2 p.c.

The blood sugar has risen 154 mg./100 c.c., so that in 1000 g. of tissue plus blood there will be 1·54 g. of free sugar, *i.e.* 1·54 g. of liver glycogen (as glucose) has been mobilized. This, for a liver of 60 g. = 2·56 p.c. liver glycogen. Adding to this the 0·2 p.c. remaining after decapitation, the initial liver glycogen was 2·76 p.c.

The other examples, similarly calculated, are shown in Table III.

It will be seen that, as a mean of four experiments, the initial liver glycogen of cats fasted for 44 hours was 2·23 p.c., while that for cats fasted 16 hours was 5·25 p.c. The figures arrived at by another method by Evans, Tsai and Young [1931 *a*, p. 74] were of about the same order. Anæsthetization with N_2O and decapitation reduced the liver glycogen of the animals with 44 hours fast to 16 p.c. of its initial value, and of those with 16 hours fast only to 34 p.c. of its initial value in spite of the fact that in the latter case more than twice as much glycogen was mobilized (2·79 g.) as in the former (1·3 g.). The three experiments (357, 358 and 359) in which the suprarenals were inactivated illustrate the fact that much smaller amounts of glycogen are mobilized and of glucose liberated on decapitation than happens with animals not so treated. The last three experiments show that in pregnancy the liver glycogen can reach quite low fasting levels, as contrasted with those late in lactation (kittens weaned) when it reaches the highest level of the whole series.

TABLE III. Calculation of initial liver glycogen.

Exp.	Length of fast hours	Body wt. kg.	Initial wt. of liver g.	Rise in total sugar of body on decapitation or decerebration g.	Loss of liver glycogen equivalent to rise in total sugar g./100 g. liver	Residual glycogen content of liver after decapitation or decerebration g./100 g.	Initial liver glycogen content g./100 g.
345. Decapitate	44	3·0	60	1·54	2·56	0·20	2·76
341. Decapitate	44	3·0	70	0·90	1·28	0·04	1·32
352. Decerebrate *post partum*	44	3·25	80	1·67	2·08	0·97	3·05
353. Decapitate	44	3·25	71	1·09	1·54	0·24	1·78
				Mean = 1·3		Mean = 0·36	2·23
343. Decapitate	16	3·5	70	3·32	4·74	0·97	5·71
346. Decapitate	16	2·5	93	2·60	2·84	2·05	4·89
351. Decapitate	16	3·25	89	2·45	2·73	2·41	5·14
				Mean = 2·79		Mean = 1·81	5·25
358. R.S.A. Decapitate	16	2·75	98	0·73	0·74	5·29	6·03
359. R.S.A. Decerebrate	44	3·75	91	1·03	1·14	0·07	1·21
357. L.R.S. L.A. Decapitate	49	2·5	68	0·80	1·18	2·72	3·90
355. Pregnant. Decerebrate	50	3·0	62	0·75	1·20	0·03	1·23
344. Pregnant. Decapitate	44	3·5	65	0·08	0·12	0·02	0·14
356. Late lactating. Decapitate	46	3·0	75	3·17	4·23	2·01	6·24

SUMMARY.

1. Anæsthesia is easily induced in a large majority of cats by means of nitrous oxide containing 5–12 p.c. of oxygen; it can be maintained for long periods; recovery is rapid, complete and quiet.

2. Blood sugar and lactate rise, on induction, to an extent which varies with individuals, nutritional state, and other conditions. In the same animal the blood sugar rises less when the store of liver glycogen is lower, *e.g.* after prolonged fasting, particularly in late pregnancy. Usually the blood sugar in animals fasted for 44 hours (initially 90 mg./100 c.c.) reaches about 126 mg./100 c.c. after 10 min., and rises thereafter to about 180 mg./100 c.c. at the end of an hour. In some cases it falls after 10–30 min. In recovery there is often a slight rise followed by a slow fall. Blood lactic acid usually rises from an initial value of 16 mg./100 c.c. to about double that amount in 20 min., after which the amount changes little or may decline.

3. Decapitation under nitrous oxide, despite the subsequent rapid elimination of the anæsthetic, showed the same series of changes in carbohydrate storage as are seen when the operation is performed under ether, over which it has no particular advantage. Decerebration, though very conveniently performed under N_2O, also gave similar results to those under ether.

4. After inactivation of the suprarenals by section of one splanchnic nerve and removal of the contralateral suprarenal the depletion of liver glycogen caused by anæsthetization with N_2O, decapitation or decerebration, was less than with suprarenals intact.

5. Calculations indicate that in cats fasted for 44 hours the liver glycogen is about 2·2 p.c., except in pregnant animals, in which it is much lower; and that in the decapitate cat glyconeogenesis is usually proceeding at the rate of at least 0·6 g. per hour.

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THE UTILIZATION OF BLOOD SUGAR AND LACTATE BY THE HEART-LUNG PREPARATION.

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Changes in sugar and lactate concentration have often been shown to occur in the blood taken from the reservoir of the heart-lung preparation, and it has been deduced from these changes that the heart regularly removes a definite amount of glucose, either for conversion into glycogen or for direct combustion. Thus Patterson and Starling [1913] found the glucose loss of the heart-lung preparation to be from 0·8 to 3·8 mg. glucose per gram of heart per hour; certain deductions, such as the amount used by the lungs, would need to be made from this. Cruickshank and Startup [1933] give much higher figures, and state that the fall of blood sugar, which nearly accounts for the oxygen utilization of the heart, amounts to about 5·28 mg./g. heart/hour; excess of sugar lost over sugar oxidized (calculated from oxygen usage) is taken by them to represent storage as glycogen. This, however, is small for ordinary conditions. Results obtained in these ways are complicated by various considerations, such as constant leakage away of blood, sugar utilization of lungs, and glycolysis or other changes in the blood itself, and it would seem desirable, if only as a check, to determine the utilization by measurement of the blood flow through the heart and direct analyses of the arterial blood entering the heart and of the coronary sinus blood leaving it. If the coronary blood flow were of the order of 1 c.c./g./min. [Evans and Starling, 1913] then, from Cruickshank and Startup's figure, this should give a glucose difference of about 9 mg./100 c.c. between the two

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bloods, while, if we take Patterson and Starling's figure at only 2 mg./g./hour, the difference would be about 3·5 mg./100 c.c., so that a heart weighing 80 g. would remove, according to these different authors, from 7 to 2·7 mg./min. respectively. Such differences in glucose content, though small, should easily be capable of detection, and of at least approximate measurement.

Lactic acid variations have been less investigated [Tsuji, 1916], though Anrep and Cannan [1923] made the fundamental observation that the lactate concentration of the blood in the heart-lung preparation rose and fell with the *p*H of the blood, when this was altered above or below about 7·4, by ventilation with air or with oxygen and CO_2, though they gave no explanation of the origin or fate of the lactic acid. Eggleton and Evans [1930*a*] showed that a similar alteration took place in the lactate concentration of the blood perfused through a limb, when the lungs used for oxygenation were ventilated in these two ways, but they also gave no final evidence as to the origin of the lactic acid.

The purpose of the present experiments, begun early in 1932, was to attempt to give a more complete explanation of the phenomenon which occurred in the heart-lung preparation. We soon became aware of the work of McGinty [1931], which, confirming that of Himwich, Koskoff and Nahum [1928], showed that the heart uses lactic acid in considerable amount. Our results with a different method fully confirm this and subsequent investigations of McGinty and his associates [1931, 1932, 1933].

Methods.

Heart-lung preparations, with defibrination, were made in the usual manner, and special precautions taken to keep the temperature constant at or near to 38° C., by having the reservoir and resistance immersed in a thermostat tank. Arterial pressure and output were also kept as constant as possible, the pressure, usually 100 mm. Hg, being in some experiments kept as low as 70 mm. Hg, in order to give a smaller coronary flow in which changes of composition should be more conspicuous. Ventilation, either with air, or with other gas mixtures of known composition, was carried out by a Starling Ideal Pump. After establishment of the preparation it was allowed to work under the chosen conditions for from 30 min. to 1 hour, in order to ensure that everything was in equilibrium before taking any samples. The Morawitz coronary sinus cannula was connected by two T-tubes to the venous inflow as was done by Evans and Starling [1913, Fig. 3), so that the sinus blood, when not being collected, flowed back to the heart. Coronary sinus flows were measured

at frequent intervals and averaged, and samples were not collected until the coronary circuit had been opened for measurement for a few minutes. Then samples of arterial blood, from the aorta, and of coronary sinus blood were simultaneously collected for analysis; observations of cardiac output, heart rate, temperature and arterial pressure were also made at the time of sampling. The total coronary flow was found by multiplying the sinus flow by 5/3 [Evans and Starling, 1913].

At each sampling two (or even four) analyses for blood sugar and blood lactate were carried out on each type of blood, and the results averaged for each. In order further to reduce errors, several such samplings, where possible, were made without changing the conditions and all the results again averaged. In order to save space in the presentation of results, which in many of the experiments involved making twelve or more analyses of each blood without change of conditions, we shall, wherever possible, confine ourselves to these average figures and present them in the form of tables. Fig. 1 shows the rate of uptake of glucose and lactic acid in one of the experiments on oxygen lack, and Fig. 2 illustrates the experimental data from which Fig. 1 was prepared.

Blood sugar analyses were in the earlier experiments made by the Hagedorn and Jensen method, but in the majority we determined true sugar by Somogyi's [1930] method of protein precipitation, followed by determination by the Shaffer-Hartmann modified method.

Lactic acid determinations were made by the method of Friedemann, Cotonio and Shaffer [1927] after deproteinization with 2 p.c. trichloroacetic acid and removal of sugar with copper and lime.

Results.

It will be evident from the last two columns of Table I that when preparations were ventilated with air, the glucose utilization by the heart was, as found by McGinty, on the average extremely small in comparison with the uptake of lactic acid. This low average result is in part due to the inclusion of several instances in which sugar was apparently added to the blood in passing the coronary vessels. These are shown in the tables by a minus sign. We have for long hesitated whether to include such examples, or whether to reject them as due to experimental error, because they seemed to run counter to all probability. Finally it seemed desirable to include them, partly on account of their individual frequency and magnitude, which would seem to preclude the likelihood that they represent erroneous estimations, and partly because, as will appear, we have seen them under other circumstances. Whether, in spite of their

occurrence when Somogyi's deproteinization method was used, they represent an output of actual glucose from the heart, or whether any other explanation, such as the scission of some higher blood carbohydrate, or the formation or liberation of some other reducing substance, is the correct one, we have not attempted to determine.

TABLE I. Ventilation with air.

Exp.	Total period of observation min.	Number of duplicate observations made	Glucose mg./100 c.c. mean of all analyses: Arterial	Glucose mg./100 c.c. mean of all analyses: Coronary	Lactic acid mg./100 c.c. mean of all analyses: Arterial	Lactic acid mg./100 c.c. mean of all analyses: Coronary	Removed per min. by heart: Glucose mg.	Removed per min. by heart: Lactic acid mg.
251	8	2	130	134	119	120	−7·00	− 0·60
255	20	2	89	107	112	84	−6·70	10·60
271	87	4	80	78	66	58	1·60	6·30
272	42	3	216	218	59	61	−1·12	− 0·95
275	15	2	106·5	107·5	112·5	87·5	−0·75	18·80
281	64	3	113·7	113·3	73·3	65·0	0·21	4·40
282	24	3	89·2	90·8	134·0	113·7	−0·40	13·80
283	20	1	13·0	11·0	20·0	19·0	2·00	1·00
285	40	3	153·3	149·3	96·3	87·3	1·70	3·90
285*	30	2	65·0	63·0	83·5	75·5	0·90	3·40
288	20	2	50·5	47·0	103·5	76·0	1·50	11·60
289	30	3	158·0	158·0	69·7	59·0	0·00	6·30
289*	35	2	85·5	83·5	70·0	68·0	2·10	2·10
289†	125	3	0·0	0·0	27·3	27·3	0·00	0·00
290	30	3	80·5	86·0	46·5	50·5	−3·10	− 2·30
290*	41	2	9·5	5·5	56·5	51·5	3·60	4·50
291	30	3	176·3	174·0	58·3	58·7	0·70	− 0·10
291*	120	4	89·0	87·7	70·8	58·3	0·80	7·50
292	30	3	95·2	93·3	61·8	59·7	0·86	0·95
292*	120	5	20·0	18·6	66·4	66·0	2·70	0·77
297	60	4	92·1	91·2	22·1	22·4	0·67	0·22
309	120	5	59·8	52·4	69·6	58·4	0·74	1·12
309‡	60	2	0·0	0·0	76·5	51·0	0·00	> 2·60
310	180	7	108·2	103·5	58·0	55·3	2·80	1·62
311	180	7	74·6	74·0	48·8	47·0	0·48	1·42
314§	30	2	72·0	72·0	38·0	32·5	0·00	3·30
315	30	2	64·0	66·0	36·0	30·5	−2·50	6·90
318	60	3	82·3	79·3	64·4	44·0	0·66	2·30
318‖	30	2	17·3	15·0	59·5	54·7	0·75	1·64
							Mean = 0·11	3·90

* Period following N_2 ventilation.
† Blood now sugar-free.
‡ End portion of long experiment. Blood now sugar-free.
§ After 10 p.c. CO_2 in O_2.
‖ After 5 p.c. CO_2 in O_2.

In this connection, although it is perhaps not fully justifiable to compare results obtained by perfusion of cold-blooded hearts with Ringer's fluid with those obtained from blood-fed mammalian hearts, it may be mentioned that Clark, Gaddie and Stewart [1931, 1932*a*] found that the perfused frog heart formed some fermentable reducing substances from a non-carbohydrate source.

One explanation for an output of sugar from the heart might be entertained as conceivable for the period immediately after establishment of the preparation. Visscher and Mulder [1930] have shown that the tissue sugar of the heart runs parallel with the sugar content of the blood; during the making of the preparation the animal's blood sugar reaches high levels, say 300 mg./100 c.c., and the heart tissue, say 80 g., gets into equilibrium with this. At completion, new defibrinated blood containing, say, 150 mg./100 c.c. glucose, is added to the circulation, thus lowering its concentration to, say, 200 mg./100 c.c. For a time glucose will now be draining out of the heart to restore equilibrium: we may suppose that if this were a uniform process our 80 g. heart, having 80 mg. of glucose to discharge, could liberate 10 mg./min. for 8 min. or any less amount for a proportionally longer period. The balance of probability, however, is that the outward diffusion of sugar would be high at first, but negligibly small after half an hour.

The preliminary period was introduced expressly to avoid equilibration changes: moreover, out of the first twenty-four experiments done, it appeared that, at the first sampling, the arterial blood sugar was higher than the coronary 11 times, and lower 13 times; at the second sampling the arterial was higher 11 times, the coronary 12 times, and once there was equality: at the third sampling the figures were 14, 6 and 4 respectively: in spite of the third series these figures, in view of the fact that the first sampling was itself preceded by a run of 30 min. to an hour, do not seem much in favour of this explanation. Also, the lactic acid figures would be expected to show a parallel phenomenon, which is not the case. In view of the fact that the table includes twenty-nine series of observations on twenty-one different preparations, we may then take the figure 0·11 mg./min. as approximating to the average rate at which glucose is removed by dogs' hearts of not less than about 90 g. weight, *i.e.* about 0·072 mg./g./hour. Even the highest individual figure in any of our experiments (3·6 mg./min.) would only represent a usage of about 2·4 mg./g./hour, and this was obtained after a period of oxygen lack, which, as the table shows, is in other instances followed by an increased glucose uptake.

It would seem then, that in preparations made acapnic by ventilation with air, the glucose usage of the heart is practically negligible.

Lactic acid was taken up by the heart in 25 out of the 29 experiments, and in the remaining four experiments the outputs of lactate from the heart into the blood were, with one exception, very small. The average rate of uptake is 3·9 mg./min. or 2·6 mg./g./hour (for 90 g. heart). It is, perhaps, worth while noting that these amounts of lactic acid and

glucose added together, *i.e.* about 2·7 mg./g./hour, would on combustion require 2·03 c.c. O_2/g./hour. For similar preparations, Starling and Evans [1914] give 3·2 c.c./g./hour, only about one-third of which they estimated to be used in burning carbohydrate, while Cruickshank and Startup give 3·9 c.c./g./hour. Allowing for no combustion other than that of glucose and lactic acid, only 72 p.c. of the oxygen usage of the heart, on Evans and Starling's figure, could be covered by the amounts of these substances shown to be taken up; whether the lactic acid was burned direct, or first converted into glycogen would of course be immaterial. Hence it would seem essential, either for the heart to draw on its own glycogen store, or else to burn substances other than carbohydrate or lactic acid. Cruickshank [1913] and Visscher and Mulder [1930] found no evidence for glycogen loss in heart-lung preparations such as these, while Lovatt Evans [1914] calculated from the respiratory quotients of heart-lung preparations that as little as 36 p.c. of the energy might be derived from oxidation of carbohydrates or their allies, and Clark, Gaddie and Stewart [1932*a*] also believe that in the frog heart, the carbohydrate metabolism only accounts for about 40 p.c. of the whole. The exact fraction of the metabolism provided by carbohydrate and/or lactic acid, as determined by the method of respiratory quotient, is, however, still somewhat uncertain, though Cruickshank and Startup found the R.Q. to be often unity and assume that the whole of the metabolism may be of the carbohydrate type.

TABLE II. Ventilation with oxygen containing up to 6·5 p.c. CO_2.

Exp.	CO_2 p.c.	Total period of observation min.	Number of duplicate observations made	Glucose mg./100 c.c. mean of all analyses: Arterial	Glucose mg./100 c.c. mean of all analyses: Coronary	Lactic acid mg./100 c.c. mean of all analyses: Arterial	Lactic acid mg./100 c.c. mean of all analyses: Coronary	Removed per min. by heart: Glucose mg.	Removed per min. by heart: Lactic acid mg.
257	5·0	40	3	142·0	149·0	70·0	53·0	−1·6	4·5
259	5·0	22	2	351·0	325·0	48·0	36·0	4·7	2·0
263	5·0	143	4	170·0	169·0	31·0	22·0	0·87	7·3
264	5·0	129	4	88·0	83·0	36·0	27·0	3·4	6·5
267	5·0	73	5	58·0	52·0	52·0	41·0	3·4	6·6
277	3·8	38	3	112·0	112·0	52·8	40·8	0·0	5·9
278	5·0	18	3	144·0	146·0	45·7	34·0	−2·2	12·8
279	5·0	60	3	131·0	130·0	62·3	53·3	0·8	6·9
280	5·0	120	4	102·8	100·0	32·2	28·7	1·95	2·45
282	5·0	57	3	19·9	19·6	81·3	72·3	0·40	11·20
287	6·5	67	3	129·0	125·0	60·3	50·7	2·2	5·4
294	5·0	15	2	178·0	175·5	33·0	27·5	1·30	2·85
294*	5·0	30	2	121·5	119·5	22·0	21·5	1·10	0·28
318	5·0	30	2	54·7	54·5	53·5	47·0	0·07	2·20
								Mean = 1·17	5·50

* Period following ventilation with 4 p.c. CO_2 + 96 p.c. N_2.

When preparations were ventilated with oxygen containing up to 6·5 p.c. carbon dioxide, the uptake of glucose was considerably and that of lactic acid slightly increased, the amounts being 1·17 and 5·50 mg./min. respectively, or (for 90 g. heart) about 0·78 mg./g./hour glucose and 3·67 mg./g./hour lactic acid, together equivalent if burnt, to an oxygen usage of 3·35 c.c./g./hour. McGinty and Miller [1933], by two different methods, with hearts probably in a condition comparable with these, found averages of 0·2 and 0·3 mg. glucose, and 1·5 and 3·1 mg. lactic acid per g. heart per hour.

When oxygen containing over 8 p.c. of CO_2 was used for ventilating the preparations the sugar uptake rose still further to an average of 1·48 mg./min., while the lactate fell again to about 3·5 mg./min. These correspond to 0·99 mg./g./hour glucose and 2·34 mg./g./hour lactic acid, or jointly 2·5 c.c. O_2/g./hour. It would seem that the greatest assimilation

TABLE III. Ventilation with oxygen containing 8 p.c. or over of CO_2.

Exp.	CO_2 p.c.	Total period observation min.	Number of duplicate observations made	Glucose mg./100 c.c. mean of all analyses: Arterial	Glucose mg./100 c.c. mean of all analyses: Coronary	Lactic acid mg./100 c.c. mean of all analyses: Arterial	Lactic acid mg./100 c.c. mean of all analyses: Coronary	Removal per minute by heart: Glucose mg.	Removal per minute by heart: Lactic acid mg.
271	19·0	42	3	142·0	139·0	46·3	40·0	4·0	8·3
272	18·0	17	2	163·0	162·5	29·1	27·9	0·96	2·3
273	8·0	35	2	172·0	169·0	65·5	47·0	1·20	7·1
283	14·4	93	3	54·3	56·0	24·3	15·3	−2·20	11·9
287*	10·8	32	2	62·5	60·0	49·0	51·0	2·0	−1·6
287*	10·8	44	2	14·5	19·0	43·0	41·5	−6·2	1·7
293	10·0	30	3	84·7	83·7	56·0	56·0	0·63	0·0
295	10·0	30	3	130·7	129·3	35·3	29·3	0·78	3·4
295†	10·0	54	2	93·0	86·5	12·0	12·0	5·1	0·0
312	10·0	150	6	100·2	96·5	32·0	27·1	2·95	3·9
314	10·0	60	3	128·3	124·3	25·3	16·6	1·6	3·4
314‡	10·0	60	2	31·0	28·0	22·0	13·0	5·8	1·7
315	10·0	30	2	110·5	109·3	37·0	32·0	1·05	4·3
315‡	10·0	60	3	19·0	16·7	16·0	13·7	3·0	3·0
								Mean = 1·48	3·50

* Period following ventilation for 12 min. with 10·4 p.c. CO_2 + 89·6 p.c. N_2 + 12 min. on 6·5 p.c. CO_2 + 93·5 p.c. O_2.
† Period following ventilation for 11 min. with 10 p.c. CO_2 + 90 p.c. N_2.
‡ Periods following ventilation with air.

was when carbon dioxide up to 6·5 (mostly 5) p.c. was supplied, and that perhaps under these optimal conditions the metabolism may be covered, or nearly covered, by combustion of carbohydrate and lactic acid.

Examination of the individual results does not show any clear relationship between the uptake of glucose or (as also found by McGinty [1931]) of lactic acid and the concentration of either substance in the

blood. In one case (Exp. 289) where the blood became sugar-free at the end of several hours, the lactate uptake fell to zero with the sugar uptake: in the other instance (Exp. 309) the lactate usage was increased as the sugar usage fell to zero. We have never had any instance where the blood was free from lactic acid.

Influence of oxygen lack.

Oxygen lack was induced by giving ventilation with nitrogen (containing about 1 p.c. O_2) or with the same nitrogen containing 5–10 p.c. of CO_2 or, in one experiment, with coal gas; also by administration of sodium cyanide. The reduction of oxygen usage by the tissue was slow when pure nitrogen was given, but almost instantaneous when cyanide was used. The results given in Figs. 1 and 2, and Table IV show, quite

TABLE IV. Effect of oxygen lack.

Exp.	Treatment	Duration of period min.	No. of observations	Glucose mg./100 c.c. Arterial	Glucose mg./100 c.c. Coronary	Lactic acid mg./100 c.c. Arterial	Lactic acid mg./100 c.c. Coronary	Removal per min. by heart: Glucose mg.	Removal per min. by heart: Lactic acid mg.
285	N_2	29	3	117·7	116·3	96·0	89·3	1·10	5·20
285	N_2	42	2	27·5	27·0	88·5	74·5	3·50	10·00
289	N_2	19	2	134·5	131·0	62·5	60·5	4·10	2·30
290	N_2	43	3	53·3	42·7	55·7	57·3	12·60	−1·90
291	N_2	10	2	156·0	149·5	69·7	83·8	4·30	−9·30
292	N_2	20	2	74·0	72·0	72·5	74·5	8·10	−8·10
							Mean =	5·75	−0·30
287	N_2 + 10·4 p.c. CO_2	12	2	94·5	87·0	63·0	64·0	10·8	−1·4
287	N_2 + 10·4 p.c. CO_2	19	2	40·5	38·5	52·5	47·0	2·7	7·4
293	N_2 85; O_2 10; CO_2 5 p.c.	70	3	49·7	47·3	32·7	33·0	2·4	−0·3
295	N_2 + 10 p.c. CO_2	5	1	122·0	117·0	26·0	26·0	9·9	0·0
							Mean =	6·45	1·42
279*	NaCN 50 mg.	1	1	17·0	16·0	26·0	33·0	2·0	−14·0
280	NaCN 13 mg.	13	2	58·3	57·7	35·3	35·3	0·75	0·0
281	NaCN 13 mg.	2	1	59·0	56·0	66·0	65·0	6·0	2·0
							Mean =	2·9	−4·0
285	Coal Gas	2	1	5·0	5·0	77·0	70·0	0·0	6·2

* 40 units insulin added previously.

regularly, an increase in glucose usage and a reduction of lactate usage, or even an addition of lactate to the blood, when oxidative processes were checked by nitrogen or cyanide. This is seen more clearly when we compare, for the various types of experiment, the average glucose and lactic acid uptake per minute for the periods before, during and immediately following the period of oxygen lack.

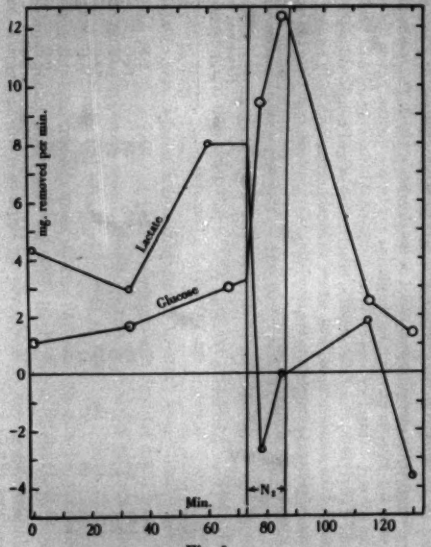

Fig. 1.

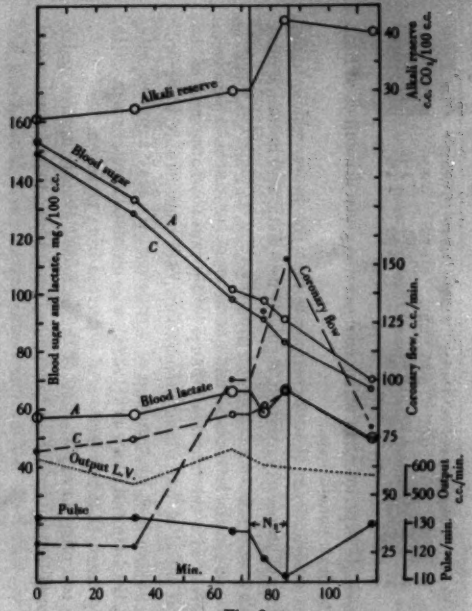

Fig. 2.

Fig. 1. Showing uptake (or output) of glucose and lactic acid by the heart in Exp. 287. Ventilation by nitrogen containing 10·4 p.c. CO_2 from 74 to 86 min. from start, before and after which ventilation was by 10·8 p.c. CO_2 in oxygen.

Fig. 2. Experimental details from which Fig. 1 was constructed: also other data.

No. of Exps.	Treatment	Mean glucose uptake mg./min. Before	During	After	Mean lactic acid uptake mg./min. Before	During	After
6	N_2	0·03	5·75	2·0	1·75	−0·30	3·65
4	N_2+CO_2	1·20	6·45	2·13	2·93	1·42	0·45
3	Cyanide	0·99	2·90	—	4·58	−4·0	—

The single experiment in which coal gas was given is insufficient from which to draw conclusions.

It is evident from these experiments that the heart, which, under conditions of proper oxygenation, regularly uses lactic acid in considerable amount, ceases to do so when its oxygen utilization is reduced or stopped, or may even, in the latter case, add quantities of lactic acid to the blood. This agrees with the finding of Tsuji [1916] that the lactic acid of the reservoir blood of heart-lung preparations rises considerably after prolonged oxygen lack by cessation of ventilation. We may suppose, with Clark, Gaddie and Stewart [1932*b*], who obtained similar results with the frog heart, that this lactic acid is derived from glycogen.

We can offer no explanation of the increased glucose uptake under these conditions; it is difficult to suppose that it is due to an experimental error.

Effect of adrenaline and of iodoacetic acid.

In two experiments adrenaline was added to the circulating blood. The results did not seem to show any pronounced effect on the glucose and lactate uptake, but as the experiments were not very satisfactory ones we do not feel justified in drawing any final conclusion from them. Tsuji [1916] found that addition of adrenaline caused a slight fall in the lactic acid content of the reservoir blood, with which we should agree.

Addition of sodium iodoacetate to the circulating blood in doses from 50 to 180 mg. was found, as the average of seven experiments, to cause an increase in an existing output of

TABLE V. Addition of iodoacetic acid.

Exp.	Dose mg.	Total time of action min.	Number of observations	Glucose mg./100 c.c. Arterial	Coronary	Lactic acid mg./100 c.c. Arterial	Coronary	Removed per min. by heart Glucose mg.	Lactic acid mg.
251*	60	5	1	66	64	34	30	2·5	4·9
255*	120	25	3	48	53	60	58	−11·0	4·5
257†	180	26	3	105	111	43	21	−10·3	35·3
273‡	100	16	1	149	168	32	35	−39·0	−6·1
275§	50	10	2	41	41	276	265	0·0	34·8
277‖	100	11	3	58·0	58·7	126·0	128·7	−1·3	−4·8
288*	80	16	1	32·0	32·0	61·0	62·0	0·0	−0·5
								Mean = −8·44	9·73

* Ventilated with air.
† Ventilated with 5 p.c. CO_2.
‡ Ventilated with 8 p.c. CO_2. Œdema followed 9 minutes after end of period.
§ Ventilated with air. 1·2 g. racemic lactic acid (as sodium lactate) added 1 hour before.
‖ Ventilated with 3·8 p.c. CO_2. 1·2 g. racemic lactic acid (as sodium lactate) added 1 hour before.

TABLE VI. Effect of adrenaline (air ventilation).

Exp.	Dose mg.	Total time of action min.	Number of observations	Glucose mg./100 c.c. Arterial	Glucose mg./100 c.c. Coronary	Lactic acid mg./100 c.c. Arterial	Lactic acid mg./100 c.c. Coronary	Removal per min. by heart: Glucose mg.	Removal per min. by heart: Lactic acid mg.
251	0·3	47	4	92	96	50	45	−4·2	4·3
255	0·1	29	2	67	82	88	84	−11·0	2·3
							Mean =	−7·6	3·3

glucose from the heart into the blood (−2·4 mg./min. to −8·44 mg./min.), and to cause no pronounced change in lactic acid uptake, which was already high (8·4 mg./min. to 9·7 mg./min.). We have not studied the effect of iodoacetic acid in conditions of oxygen lack, because the experiments with it are attended with two drawbacks which vitiate the results. First, the coronary blood flow shows such an enormous increase that accurate measurement becomes difficult; the rate of flow was always increased about sevenfold, and was the largest augmentation we have ever witnessed, whereas adrenaline only about doubled or trebled the flow: second, though the addition of iodoacetic acid to the heart seemed to produce no deleterious effect, lung œdema becomes evident after a few minutes and preliminary changes even before then probably influence the result.

Origin of the blood lactic acid.

It seems certain, since, except under conditions of extreme oxygen lack, the heart always removes lactic acid from the blood, that the lactic acid must be formed either in the blood itself, or in the lungs, or perhaps in both. At or below a critical *p*H (7·4) of the blood, Anrep and Cannan found that the lactic acid content of the store of blood in the venous reservoir nearly always falls, while above this *p*H it sometimes rises. This means that the rate at which lactic acid is produced in the preparation is reduced, or else that the rate at which it is removed by the heart is accelerated, when the *p*H of the blood is lowered beyond about 7·4. Our experiments on the effect of ventilation of the lungs with CO_2-oxygen mixtures containing over 8 p.c. CO_2 give no grounds for belief that the responsible factor in causing the fall of lactic acid can be an accelerated rate of removal by the heart, and leave us with the probability that it must be due to a reduced rate of formation. And, in fact, most of the biochemical processes which result in the formation of lactic acid are accelerated by rise and retarded by fall of *p*H.

Since the heart itself, even when acapnic, can now be excluded as a possible source of lactic acid, the most likely origin of the lactic acid is, of course, formation from glucose by glycolysis in the blood itself. It has been shown by Mellanby and Thomas [1920] and by Lovatt Evans [1922] that this process is accelerated by removal of carbon dioxide, or by otherwise increasing the *p*H of the blood. As it is essential, before

accepting glycolysis as the source of the lactic acid in our present experiments, to know whether the rate at which it proceeds is adequate to explain the phenomenon in question, we made some further experiments in order to determine the average rate of glycolysis in defibrinated dog's blood under appropriate conditions.

In these experiments blood, defibrinated in the usual way, was caused to circulate as rapidly as possible, by means of a Dale and Schuster pump [Dale and Schuster, 1928], round a circuit containing an oxygenator [v. Euler and Heymans, 1932]; the whole was kept at 38° C., the oxygenator was fed with a constant current of air, oxygen or CO_2-oxygen mixture, and samples of the blood were removed at intervals for glucose and lactic acid determinations. When the oxygenator was supplied with air or oxygen, the blood reached a *p*H of about 7·8, and its plasma had a bicarbonate value of about 18 volumes/100 c.c.: on 10 p.c. CO_2 the values were *p*H 7·2 and about 50 volumes/100 c.c. respectively. Somewhat similar experiments have been carried out by Cruickshank and Startup [1932].

It was found that glucose loss and lactic acid accumulation occurred in all the experiments, whether CO_2 was added to the gas used for aerating the blood or not, and in seven experiments which were performed the results were as uniform as could be expected. Fig. 3 shows the mean results of these experiments, from which it appeared that glucose loss and lactic acid formation took place at the following average rates:

	Change per hour mg./100 c.c.		Glucose lost appearing as lactic acid p.c.
	Glucose	Lactic acid	
Aerated with air	−18·6	+14·3	77
Aerated with 10 p.c. CO_2	−11·1	+ 9·4	85

The rate of loss of sugar was comparable with that found by Macleod [1913].

It will be seen that we failed to confirm the finding of Cruickshank and Startup that 70 p.c. of the sugar which disappears does not appear as lactic acid, and can only suppose that there must be some difference in the way in which the experiments were carried out. We think that the rate of change we have found is in agreement with that found by others [*e.g.* Anrep and Cannan, 1923, or Häusler, 1927], and that it may be taken provisionally as representing the usual extent of glycolysis in defibrinated dog's blood.

We may now proceed to estimate the relative rates of lactic acid formation and removal in the heart-lung preparation, under the conditions of our experiments. The total volume of blood in circulation in the preparations was on an average 800 c.c., and leakage was reduced to a minimum. This, as will be shown, is a point of importance. In the experiments with air ventilation summarized in Table I, there would therefore be an average hourly loss of sugar from the 800 c.c. of blood of about

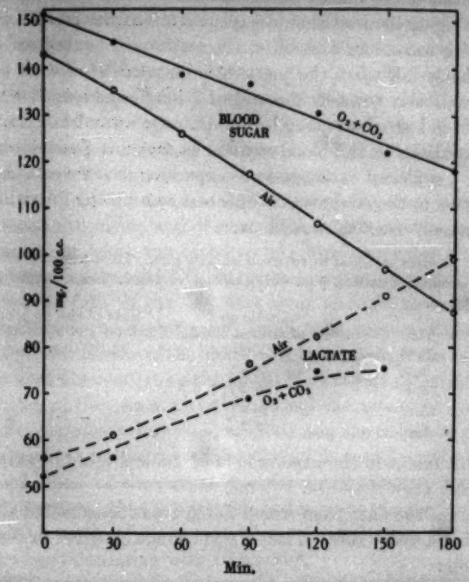

Fig. 3. Rate of loss of glucose and of formation of lactic acid in defibrinated dog's blood ventilated with air and with 10 p.c. CO_2 in oxygen. Mean of seven experiments.

149 mg. by glycolysis, plus about 7 mg. which is removed direct by the heart, making a total of 156 mg./hour. This agrees with the loss we actually found, and also with that obtained by Visscher and Mulder (139–368 mg.). At the same time there is formation, by glycolysis, of about 114 mg./hour of lactic acid, while the heart, as averaged in Table I, removes 234 mg./hour. Hence, if lactic acid is only formed in the blood, in the way we have supposed, there should be, on the average, not a gain

of lactate at all, but a loss of 120 mg./hour, or, roughly, a fall of lactic acid concentration in the reservoir blood of about 15 mg./100 c.c./hour. Now, it will be evident that whether the lactic acid concentration of the blood falls or rises will depend on the relative rates at which it is formed and removed: hence, supposing glycolysis to proceed at approximately the same rate, and the uptake by the heart to be the same for unit weight and time, it is clear that the determining factor is the mass of the heart relative to that of the blood. When the blood volume is small relative to the heart weight, then we should expect a fall of the lactate concentration, and *vice versa*. And actually, the lactic acid content of the blood does frequently fall when the preparation is ventilated with air; this is especially well seen towards the end of a long experiment, when much blood has been lost in sampling, or by drainage away by leaks. We have avoided variations in the blood volume as much as possible, but it has occurred to a sufficient extent in some experiments to reverse the effect of air ventilation in the later stages. This is shown by the following extract from a protocol, selected at random:

Exp. 291. Volume of blood in reservoir at 3.03 p.m. = 770 c.c. Ventilation with air. Experiment terminated at 6.55 p.m. owing to lack of blood. Heart weight 92 g. Heart glycogen 0·133 p.c.

Time p.m.	Lactic acid of arterial blood mg./100 c.c.	Rate of rise (+), or fall (−), of lactic acid mg./100 c.c./hour
4.00	50·6	+13·2
4.30	57·2	
5.25	75·9	− 5·1
6.55	68·2	

The variable factor in the experiments of Table I was the weight of the hearts, rather than the blood volume.

Analysis of the data from which Table I was constructed shows that the following average rates of lactic acid change occurred in the blood of the reservoir:

In nine experiments the lactic acid rose, at average rate of 27·3 mg./100 c.c./hour.

In nine experiments it fell at average rate of 60 mg./100 c.c./hour.

In three experiments there was no significant change.

Average of the twenty-one experiments = rise of 14·7 mg./100 c.c./hour.

This average agrees so well with the estimated figure of 15 mg./100 c.c./hour as to leave no reasonable doubt that lactate is formed only in the blood, and that the lactate concentration in the heart-lung blood at any time represents the balance between production by glycolysis and removal by the heart. Hence, when the volume of blood is great relative

to the weight of the heart, ventilation with air results in a rise of lactic acid content.

Turning now to the conditions when the ventilation was effected by mixtures of oxygen and carbon dioxide, the results of the glycolysis experiments already referred to show that when the blood was exposed to 10 p.c. CO_2, only about 9·4 mg./100 c.c./hour of lactic acid was formed. Hence, when the heart-lung preparation is ventilated in a similar manner, about 75 mg./hour would be formed when there was 800 c.c. of blood in circulation. The heart, according to Table III, is meanwhile removing lactic acid at the rate of 210 mg./hour, so that there should be a loss of lactic acid of 135 mg./hour, or 16·8 mg./100 c.c./hour. Actually, in the experiments from which Table III was made, the rate of fall was 17·6 mg./100 c.c./hour, while for those of Table II, in which weaker CO_2 concentrations were used, the rate of fall was 12·6 mg./100 c.c./hour, both of which figures agree well with our calculations.

It will be obvious, since administration of CO_2 does not stop, but merely retards, glycolysis, and in view of the considerations regarding the relative masses of heart and blood to which reference has already been made, that ventilation with CO_2 mixtures need not necessarily cause the blood lactate to fall. If the CO_2 were weak enough, the blood volume large enough, or the heart sufficiently small, then, in spite of the condition of ventilation, the lactate of the blood would rise, though less rapidly than if air were used for ventilating the lungs. We have no example to offer as an illustration of this, since we have not had the necessary conditions in any of our experiments, but we have often seen the tendency show itself. With hearts of the weight we commonly had, it would require more than 2·2 litres of blood in circulation in order for the lactate formation to balance the removal; but suppose we had a heart of only 50 g., and 2·1 litres of blood, a rise of blood lactate of 3·6 mg./100 c.c./hour would be predicted when ventilating with 10 p.c. CO_2.

We still have to consider the possible rôle of the lungs in connection with the formation, or removal, of lactic acid. It was found by Eggleton and Evans [1930*a*] that no disappearance of lactic acid from the blood resulted when it was circulated by a pump through the lungs, even when these were ventilated with oxygen containing CO_2. One of their experiments [1930*a*, p. 262], in fact, suggested that on passage through the lungs the blood lactic acid accumulated even more rapidly than in the present experiments where oxygenation took place with an artificial oxygenator, viz. 52 mg./100 c.c./hour on air and 18 mg./100 c.c./hour on 10 p.c. CO_2.

We have not attempted to repeat this experiment, but we did make three experiments in which samples were simultaneously taken from the right and left ventricles of a heart-lung preparation. These showed no significant difference between the two bloods: of course, in view of the rapidity of the blood flow through the lungs, the method could not show the occurrence of very small changes, which nevertheless might represent appreciable quantities per minute. However, we saw no reason to pursue this aspect of the question any further, since we were satisfied that glycolysis in the blood itself could account for the lactic acid formation in the heart-lung preparation.

Relation of these results to the origin of lactic acid in the intact body.

Our results have, we believe, some further application. Lactic acid, in amounts of about 10–15 mg./100 c.c. during rest, is a normal constituent of human blood, and occurs in very similar concentration in the blood of other mammals. It has been assumed, though without evidence, that this lactic acid represents a slow leakage away of the substance from muscles: the diaphragm and the heart have been invoked, as muscles which are always in rhythmic activity, as the source of it. We must now exclude the heart, and instead regard it as an organ by which lactic acid is rapidly removed from the circulation. The liver also, as shown by Eggleton and Evans [1930*b*], rapidly clears the blood of lactic acid after exercise. Resting muscle may also be regarded as a source of loss, rather than gain, of blood lactate. But it might be, and indeed has been, supposed that a slow formation of lactic acid, associated with the chemical changes connected with the maintenance of tonus in the "resting" skeletal muscles, is the source from which the blood derives its lactic acid during bodily rest. This rests on the assumption that the lactic acid of the resting muscle is equal to that of the plasma. But we do not know precisely what the lactate concentration of resting muscle is: quite possibly it is below that of the plasma. But the clear-cut demonstration by Owles [1930] that the blood lactate is not even augmented during exercise until this reaches a certain critical level (at an oxygen consumption of 1·8 litres/min. or about 6 times the resting value) makes this supposition quite inacceptable. That during severe exercise lactic acid passes out from muscle in considerable amount into the blood, will, we believe, be admitted by everyone.

It seems safe to conclude then, that in the resting state, and even under conditions of moderate exercise, the muscles allow no lactic acid to escape into the circulating blood, and we therefore propose as a more

suitable hypothesis that it is in the blood itself, by glycolysis, that the normal lactic acid in the blood during rest originates[1]. An increase of the lactic acid of the blood on over-ventilation has often been shown (*e.g.* by Macleod and Knapp [1918]; Eggleton and Evans [1930*a*]), and we must now suppose that this is produced in the same way as in our heart-lung preparations. It would be probable, if glycolysis occurs at the same rate in circulating human blood as happened in defibrinated dog's blood, that about ½ g. of lactic acid is produced hourly in the total blood of the body; most of this would be removed by the heart and liver, and a little perhaps by the skeletal muscle. The evidence for conversion of blood lactic acid into muscle glycogen is, however, in our opinion, not very convincing, and if it occurs at all is relatively slow. Since only a fraction of the whole blood at each circulation passes through the heart and liver, the failure of these to keep the blood lactate below about 12 mg./100 c.c. is intelligible. The glucose lost by glycolysis we suppose of course, under normal conditions, to be replaced by conversion of liver glycogen.

Our views on the cycle of carbohydrate in the resting body would therefore be expressed schematically as follows:

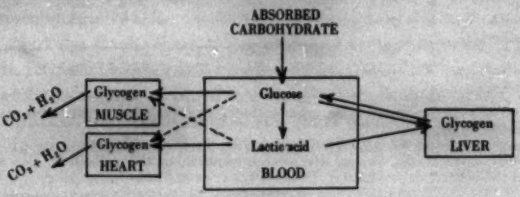

Fig. 4.

In severe exercise, or when adrenaline is being liberated in large amount, there would, of course, be a large liberation of lactic acid from the muscles into the blood stream.

Our results show that, although the glucose which disappears from the reservoir blood is ultimately utilized by the heart, this only happens to a limited extent, or often not at all, in the form of a direct uptake of the glucose by the heart tissue, but is brought about indirectly, by the conversion of the glucose first into lactic acid, which is then taken up by the heart. What its fate there may be we have no means of judging, but since the glycogen content of the heart is believed not to fall under

[1] We understand that L. C. Cook and R. H. Hurst have, in a paper now in preparation, arrived at similar conclusions.

ordinary conditions, we suppose that it is either burned directly, or else that it replaces an equivalent amount of glycogen which is oxidized.

Blood phosphate changes in the heart.

In seven of the preparations comparisons were made of the acid-soluble organic and inorganic phosphorus of the blood entering and leaving the heart. The methods used were the same as were used by some of us [De Graff, Evans and Vacek, 1932], and the results are given in summarized form in Table VII. In two cases adrenaline, and in all seven iodoacetic acid was added to the blood at the end of the experiment.

TABLE VII. Blood phosphorus in arterial and coronary blood.

		Blood phosphorus mg./100 c.c.						Removed by heart	
		Total		Inorganic		Organic			
Condition	No. of samples	Arterial	Coronary	Arterial	Coronary	Arterial	Coronary	Glucose mg./min.	Lactic acid mg./min.
Normal*	17	31·4	31·1	5·5	5·3	25·9	25·8	−0·2	5·7
Normal	4	34·8	33·8	4·6	4·0	30·2	29·8	−3·5	1·5
Same, with adrenaline†	5	28·7	30·4	4·1	4·6	24·6	25·8	−4·4	1·5
Same, later	7	33·8	33·8	4·0	3·7	29·8	30·1	−10·6	7·9
Same, after iodoacetate‡	7	30·0	30·6	3·4	3·9	26·6	26·7	−8·4	9·7

* Mean of experiments on seven preparations. In five of them ventilation was effected by 5 p.c. CO_2 in oxygen.

† Doses of 0·3 and 0·1 mg. added to reservoir. Two experiments.

‡ Seven preparations, two of which were common to the preceding. No adrenaline added to the other five previously. Doses of I.A.A. as in Table V.

In the preparations to which no additions had been made to the blood, it would seem that no significant difference is detectable between the arterial and coronary blood. In the other instances, the small number of experiments and unsatisfactory nature of the conditions, especially with iodoacetic acid, make it essential to draw none but the most tentative conclusions. Perhaps the results suggest that under the action of adrenaline there is an output of phosphorus in both forms into the blood, and this might be interpreted as indicating a breakdown of creatine phosphate, which is replaced in the later stages. Iodoacetic acid would seem to have caused little change, and that of the same nature as was produced by adrenaline. In view of the large coronary flows in both instances, however, the differences must necessarily be very small, and we therefore abandoned this part of the investigation.

Conclusions.

1. The uptake of glucose and lactic acid by the heart was investigated by the analysis of arterial and coronary samples in heart-lung preparations. An explanation is given for the rise and fall of blood lactate which occurs when the *p*H of the blood is raised or lowered.

2. When ventilation is effected with air, the heart (of 90–100 g.) removes from the blood on the average 7 mg. glucose and 234 mg. lactic acid per hour (confirming McGinty). At the same time blood sugar is being converted into lactic acid by glycolysis, at the rate of 14·3 mg. lactic acid/100 c.c./hour.

3. When the preparation contains 800 c.c. of blood, so that 114 mg. of lactic acid is formed in it per hour, there should be on the average, a fall of lactic acid, at the rate of about 15 mg./100 c.c./hour. Our experiments actually gave, on the average, a fall of 14·7 mg./100 c.c./hour. With smaller volumes of blood the rate of fall is greater.

4. When the heart is smaller, takes up lactate less readily, or the volume of blood is greater, the lactic acid of the reservoir blood may rise.

5. When the preparation is ventilated with oxygen and CO_2 the blood lactate usually falls, because the rate of glycolysis is slowed by the fall in *p*H of the blood: there is also probably a slight increase in the rate at which lactic acid is taken up by the heart. But if the heart were very small, and the volume of blood very large, then, in spite of CO_2 ventilation we predict that the lactate of the blood would rise.

6. No appreciable amount of lactate appears to be removed by the lungs.

7. It is suggested that the normal resting blood lactic acid also arises by glycolysis, and is not produced by muscle, as often supposed. This lactate is being constantly removed by the liver, and heart, and perhaps also by the muscles. Hence, as in the heart-lung preparation, the end result is that the glucose converted in the blood stream to lactate is utilized, though not directly, by the heart and other tissues.

8. The acid-soluble inorganic and organic phosphorus showed no detectable change as the blood passed the coronary circulation.

9. Adrenaline and iodoacetic acid appear to cause an output of sugar from the heart, while iodoacetic acid causes no change, or an increased uptake, of lactate.

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THE FORMATION OF LIVER GLYCOGEN IN THE CAT, UNDER VARIOUS CONDITIONS, FOLLOWING INFUSION OF AMMONIUM LACTATE.

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The original purpose of these experiments was to attempt to demonstrate the formation of liver glycogen from blood lactate in the cat under experimental conditions. Since disturbances of acid-base equilibrium are produced by the injection of lactic acid into the circulation, and, if synthesis of glycogen occurred, would also follow when sodium lactate was injected, it was thought worth while attempting the injection of the lactic acid in the form of ammonium lactate. The hope was that the substance would be converted in the liver into urea and glycogen, leaving the acid-base equilibrium of the blood and tissues unchanged. Although some glycogen was in fact laid down in the liver after the injection of ammonium lactate into the circulation, it has not been shown that an extra amount of urea is formed at the same time. The question of the fate of the ammonia has not been followed further, because interest was centred on the conditions under which glycogen became increased in the liver. The number of experiments carried out was small, and the fluctuations, as might be expected, were large, but as no further experiments are possible for some time, the results are published in this preliminary form, since they are suggestive, if not convincing. Briefly, these were that when, in order to make injections of the lactate into the portal vein, the spleen was previously removed, and the cannula for injection tied into the splenic vein, little or no increase of liver glycogen resulted from the injection. But when the spleen was left *in situ*, and the injection made into the superior mesenteric vein by a hypodermic needle passed through its wall, definite increases of liver glycogen were found after the injections. The work of certain authors,

who claimed that splenectomy caused alterations in carbohydrate metabolism, came to mind. Thus Fuziwara [1933] found that splenectomy reduced liver glycogen formation from glucose, though Togawa [1920] had concluded that it caused reduction of glycogen storage. Most of the investigations have been concerned with the blood sugar changes: a partial list of these works has been given by Rubinstein [1932]. The possible relation of the spleen to the production of acetyl-choline, and the fact that some investigators had reported that either spleen extracts [Rathery and Cosmulesco, 1933] or acetyl-choline [Ernould, 1931] caused effects on carbohydrate metabolism was further stressed by the investigations of Crocetta [1932]: this investigator concluded that acetyl-choline is expressed into the circulation when the spleen contracts.

In a further stage of the present work it was found that although removal of the spleen reduced or abolished the formation of liver glycogen after injection of ammonium lactate into the blood stream, the simultaneous or subsequent infusion of small doses of acetyl-choline resulted in glycogen deposition at a rate at least equal to that in experiments in which the spleen remained *in situ*.

Methods.

Except where otherwise stated the cats were allowed to fast for 36 hours prior to use. Under light ether anæsthesia an intravenous injection of 0·075 g./kg. of chloralose was given, after which the ether was discontinued, and an interval of an hour allowed to elapse in order to establish a steady state. Then samples of carotid blood were taken for glucose and lactic acid estimations, and duplicate liver samples taken for glycogen. The bladder was emptied and the sample of urine kept for ammonia, urea, lactate and sugar estimations. Infusion of the lactate solution was made into the appropriate vein, the splenic (with previous splenectomy) or superior mesenteric. The acetyl-choline solution in 0·9 p.c. NaCl was injected into the splenic or superior mesenteric vein. For the infusion of the lactate the slow infusion apparatus described by Burn and Dale [1924] was used: for the acetyl-choline a fine burette was used, and the injection made either into the superior mesenteric vein after the lactate infusion, and over a period of 5–10 min., or, in other cases, through a cannula in the splenic vein at intervals during the period of the lactate infusion, or for 5–10 min. after its conclusion. The amount of lactate given was about 500 mg./kg., and at a rate of about 0·2 c.c./min. of an approximately 10 p.c. solution. The lactate solution was made by neutralizing with concentrated ammonia (to alizarin) a 10 p.c. racemic

lactic acid solution (boiled under reflux for 6 hours to convert any anhydride), the neutralization always being made just before use. The exact strength of the lactate was determined by analysis each time a fresh stock of the diluted boiled lactic acid was made. The dose of acetyl-choline injected was from 0·002 to 0·06 mg. It was found impossible to give the lactate solution more rapidly than 0·3 c.c./min., as this tended to cause restlessness or even convulsions; hence the infusion of lactate occupied at least two hours, or even longer. About 3 hours after the termination of the infusion, blood and liver samples were again taken, and the urine collected as before. Methods of analysis were as described by Evans, Tsai and Young [1931] and Evans, Murphy and Young [1932].

Results.

Thirty experiments were performed in all, and the results are given in tabular form. Table I shows the results of the lactate infusion into intact cats. Whereas under chloralose alone the glycogen either falls [Murphy and Young, 1932], or, as in Table II of the present paper, in which control infusions of 0·9 p.c. NaCl were given, remains almost unchanged, yet after the lactate infusion there was in four out of five experiments a rise in liver glycogen. There was probably some unrecognized cause for the large fall in one experiment, but including all experiments the average of the series showed an increase of 0·2 g./100 g.

Table I. Infusion of ammonium lactate into superior mesenteric vein (spleen *in situ*).

No. and length of fast (hours)	Liver glycogen		Change in liver glycogen g./100 g.	Blood sugar mg./100 c.c.		Blood lactate mg./100 c.c.	
	Initial g./100 g.	Final g./100 g.		Before	After	Before	After
1. (18)	1·66, 1·46 → 1·56	2·02, 1·94, 1·93, 1·82 → 1·92	+0·36	259	212	20	21
3. (36)	3·74, 3·61 → 3·67	1·69, 2·85, 2·54 → 2·36	−1·31	190	141	18	64
4. (60)	1·04, 1·03 → 1·04	1·71, 2·10, 1·71, 1·78 → 1·82	+0·78	124	114	22·5	20
5. (60)	0·314	1·33, 1·05, 1·59, 1·46 → 1·35	+1·04	137	102	27	20
6. (36)	0·46, 0·53 → 0·50	0·73, 0·64, 0·56, 0·63 → 0·64	+0·14	153	135	13	10

Mean = +0·2 g./100 g.

TABLE II. Infusion of 0·9 p.c. NaCl into superior mesenteric vein (spleen *in situ*).

All cats fasted 36 hours.

No.	Liver glycogen: Initial g./100 g.		Liver glycogen: Final g./100 g.		Change in liver glycogen g./100 g.	Blood sugar mg./100 c.c.: Before	Blood sugar mg./100 c.c.: After	Blood lactate mg./100 c.c.: Before	Blood lactate mg./100 c.c.: After
28.	1·02 1·02	1·02	1·17 0·71 0·78 0·81	0·94	−0·08	181	124	13	23
29.	0·92 0·80	0·86	1·47 0·96	1·21	+0·35	206	136	16	15
30.	1·15 1·24	1·19	0·75 1·03 0·85 0·68	0·83	−0·36	193	155	12	11

Mean = −0·09 g./100 g.

The number of experiments as controls, shown in Table II, is too small to give significant results, but it may be conceded as improbable that the injection of 0·9 p.c. NaCl would be likely to cause an *increase* of liver glycogen.

The cats were of about 3 kg. body weight, and if we assume the lactate to have been uniformly distributed throughout one-third of the body weight, the addition of 0·5 g. of lactic acid per kg. should have given a rise of blood lactate of the order of about 150 mg./100 c.c. Since, in every case in which liver glycogen rose there was not a rise but a fall of blood lactate, we must believe that both the *d*- and *l*-forms had been either converted into glycogen, excreted, or stored somewhere in the body. The amount of urine formed in the course of and following the infusion was never great, and lactate estimations were only done on the samples in one of the experiments: this showed an excretion of only 9 mg. of lactic acid out of 1·89 g. infused, so that both the forms of lactic acid would seem to have disappeared.

If glycogen were formed from the whole of the lactate, there should have been an increase of about 1·6–1·7 g./100 g. in the liver glycogen. The increase on the average in Table I, excluding Exp. 3, was 0·58 g./100 g. This does not necessarily mean that all the glycogen formed arose from lactate, however, since it is quite possible that, as shown by Long and Horsfall [1932] for muscle, the *d*-lactate may carry with it, for conversion into glycogen, some glucose which would not otherwise have been used, and it should be noted that the blood sugar did fall in the period after the infusion.

In Table III is shown the results of the infusions of lactate into cats, carried out precisely as for those of Table I, except that the spleen was re-

moved prior to making the injection. It will be seen that in no case was there an increase of more than 0·19 g./100 g. liver glycogen, while in some considerable loss occurred. The average result is not statistically significant

TABLE III. Infusion of ammonium lactate into superior mesenteric or splenic vein (spleen removed).

No. and length of fast (hours)	Liver glycogen				Change in liver glycogen g./100 g.	Blood sugar mg./100 c.c.		Blood lactate mg./100 c.c.	
	Initial g./100 g.		Final g./100 g.			Before	After	Before	After
18. (36)	1·08 1·52	1·30	1·58 1·47 1·19 1·22	1·36	+0·06	141	138	5	73
19. (36)	1·79 1·71	1·75	1·98 2·02 2·00 1·76	1·94	+0·19	183	154	23	14
20. (36)	0·38 0·46	0·42	0·42 0·37 0·24	0·34	−0·08	156	175	12·5	46
21. (36)	1·36 1·38	1·37	1·40 1·35 1·47 1·39	1·40	+0·03	147	121	34·5	22·3
23. (36)	1·27 1·23	1·25	0·95 1·79 1·48 1·17	1·35	+0·10	152	122	21·9	15·5
24. (36)	1·42 1·46	1·44	1·33 1·17 0·93 0·85	1·06	−0·38	224	99	23	9
25. (36)	1·91 1·97	1·94	1·63 1·63 1·15 1·45	1·46	−0·48	143	262	12·5	18·6
26. (36)	0·051 0·048	0·05	0·03 0·03 0·05 0·03	0·04	−0·01	152	151	32	83

Mean = −0·07 g./100 g.

in view of the divergence of the individual values. Mere removal of the spleen from circulation, according to Evans, Murphy and Young [1932], does not interfere with the recovery of liver glycogen following decapitation, and if this is so, and if the present experiments are dependable, we must suppose that the liver glycogen formed after decapitation does not all arise from blood lactate. In some of the experiments of Table III the blood lactate is higher at the close of the experiment than it was at the commencement, though the rise is not so great as it would be calculated to be had there been no destruction or excretion of lactate.

When the animals were splenectomized, and the lactate infusion accompanied or followed by infusions of acetyl-choline into the portal circulation, there was in 6 experiments out of 8 (Table IV) an increase of liver glycogen of much the same order as that shown by the intact cats

TABLE IV. Infusion of ammonium lactate and acetyl-choline into superior mesenteric vein (spleen removed).

All cats fasted 36 hours.

No.	Acetyl-choline dose and time given	Liver glycogen g./100 g. Initial		Liver glycogen g./100 g. Final		Change in liver glycogen g./100 g.	Blood sugar mg./100 c.c. Before	Blood sugar mg./100 c.c. After	Blood lactate mg./100 c.c. Before	Blood lactate mg./100 c.c. After
9.	0·004 mg. in 10 min. after lactate	0·66 0·69	0·68	1·55 1·65 1·87 1·65	1·68	+1·00	170	116	8·4	7·1
11.	0·013 mg. in 3 periods of 10 min. after lactate	0·50 0·51	0·50	1·23 1·37 1·45 1·37	1·35	+0·85	135	166	19·6	42
12.	0·004 mg. simul-taneously	0·84 0·79	0·81	1·11 1·07 1·35 1·36	1·19	+0·38	199	208	24	32
13.	0·008 mg. simul-taneously	0·86 0·74	0·80	1·31 1·28 0·92 0·97	1·11	+0·31	111	145	6	7
15.	0·06 mg. in 10 min. after lactate	0·44 0·39	0·41	1·34 1·28 1·16 0·97	1·19	+0·78	197	159	14·9	8
17.	0·06 mg. after	0·74 0·73	0·74	1·60 1·68 1·33 1·73	1·59	+0·85	179	159	21	16

Mean +0·69 g./100 g.

infused with the lactate. Two experiments showed a fall, but these experiments were unsatisfactory, in various ways. In one of them the acetyl-choline administration *preceded* the infusion of the lactate, and the experiment was further disturbed by the fact that the animal was in an extremely excitable state: in consequence of this the needle used to infuse the solution passed through the wall of the superior mesenteric vein, causing loss of a considerable quantity of blood as well as of an unknown amount of the lactate solution. In the other the cat was remarkably bloodless: the lobes of liver when cut showed little hæmorrhage, and the liver tissue was very soft and spongy. These experiments are omitted from the table. With these exceptions, the increases of liver glycogen

were quite definite. It should be stated that the infusion of the acetyl-choline, at the slow rate employed, caused no definite change in the systemic arterial pressure.

In order to exclude the possibility that the changes in glycogen content of the liver might have been due to alteration in its water content, determinations of the latter were made in a number of experiments. The results, given in Table V, show that this factor may be discounted.

TABLE V. Changes in water content of liver.

No.	Experiment	Loss of weight at 110° C. Sample at start p.c.	Loss of weight at 110° C. Sample at end p.c.	Change in water content during experiment
6.	Lactate infusion	72·0	72·7	+0·7
8.	Lactate, spleen removed acetyl-choline	70·5	70·6	+0·1
19.	Lactate, spleen removed	76·0	70·0	−6·0
20.	" " "	63·0	56·0	−7·0
21.	" " "	70·6	71·0	+0·4
23.	" " "	69·5	71·5	+2·0
24.	" " "	70·3	71·0	+0·7
28.	Saline infusion	69·0	69·5	+0·5
29.	" "	64·5	63·4	−1·1
30.	" "	62·8	62·7	−0·1

Mean = −0·98 p.c.

CONCLUSIONS.

Formation of liver glycogen occurs in cats with all organs intact after the infusion of ammonium lactate into the superior mesenteric vein. This formation is absent or significantly reduced if the spleen is removed from the circulation. Under these conditions, however, infusion of acetyl-choline with or after the lactate leads to an increase of liver glycogen of the same order as that seen in intact cats.

I wish to express my thanks to Prof. C. Lovatt Evans for the facilities and guidance which he has accorded me.

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INFLUENCE OF THE AUTONOMIC NERVES ON ALIMENTARY HYPERGLYCÆMIA AND ON THE ABSORPTION OF GLUCOSE.

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The immediate source of the sugar causing the hyperglycæmia during absorption of carbohydrates has received a good deal of attention in recent years. The problem is one which does not at present lend itself to direct attack; for it is not possible to earmark the glucose molecule so as to render it readily recognizable after absorption, as has been done so successfully in the case of fats. Indeed, we do not even know with any degree of certainty the precise chemical nature of blood glucose.

The question has been approached by previous workers, mainly by observing the effects of blocking the autonomic nerves on the blood sugar curve after oral and intravenous or subcutaneous administration of sugar. Generally, ergotoxin or atropine or both drugs together have been employed in preference to nerve section, to cut off the impulses carried by the vagi and splanchnics. While the two drugs given simultaneously have always suppressed alimentary hyperglycæmia, singly their effects have been inconstant. In the experiments so far conducted the precautions taken to control gastric motility have not been adequate, and little or no attention has been paid to the amounts of sugar absorbed. It is obvious, as absorption of sugar occurs in the intestine and not in the stomach, that suppression of hyperglycæmia after oral administration of sugar might be attributed, in some measure, to delayed emptying of the stomach, unless it were shown that motility of the viscus was unaffected by the drugs. Furthermore, other known or possible properties of the drugs seem to have received little or no consideration, the results being attributed to their nerve-paralysing actions alone. Clearly one cannot conclude, because the most striking effects of ergotoxin and atropine are the inhibition of sympathetic and vagal impulses respectively, that the suppression of hyperglycæmia which they elicit is due

to this cause and to none other. Before such an inference could be drawn it would have to be shown, either that similar effects follow section of the nerves or that, for example, the depressing effect of ergotoxin on the circulation or of atropine on secretory cells has no influence on blood sugar.

In the experiments described in this paper the factors just enumerated were controlled in so far as possible under the experimental conditions. The main lines of study were:

(*a*) the influence of ergotoxin plus atropine on blood sugar after oral administration of glucose to normal animals;

(*b*) the effect of the drugs singly and together on the rate of discharge from the stomach and on intestinal absorption of glucose solutions in normal animals;

(*c*) the effect of the drugs singly and together and of vagotomy or splanchnotomy on blood sugar after injecting glucose into the intestine in anæsthetized animals.

Amongst other things, it was found that the cutting-off of the nervous impulses, either by atropine or by section, increased both hyperglycæmia and the absorption coefficient when the sugar solution was placed in the intestine, while ergotoxin alone or with atropine stimulated absorption but temporarily suppressed hyperglycæmia. Further experiments were therefore undertaken in the hope of throwing light on this action of the drugs, the procedures employed being to study their influence on the portal and systemic blood sugar and on glycogen formation in the liver. These experiments had an additional interest in view of the theories of Hetenyi and Pogány [1928] and of Pollack [1929]. The former maintained that impulses causing hepatic glycogenolysis are normally carried by the sympathetic, and the latter that both the splanchnics and the vagi normally convey impulses which inhibit glycogen formation. According to either of these theories, suppression of hyperglycæmia by ergotoxin and atropine should be accompanied by increasing deposition of glycogen in the liver, and probably also by little or no diminution in portal hyperglycæmia. The evidence obtained on these points was far from satisfactory.

Suppression of alimentary hyperglycæmia by ergotoxin and atropine.

These experiments were an exact repetition of Pollack's and his procedure was strictly adhered to. Typical results are shown in Fig. 1. Pollack's findings were completely confirmed and there can be no doubt that ergotoxin and atropine, when injected in the amounts and at the

times recommended by him, prevent the development of hyperglycæmia during absorption of glucose. In all subsequent experiments on rabbits and cats, the drugs were always injected according to Pollack's plan, and, when both drugs were given to the same animal, different sites of injection were selected. While the rabbit was the most suitable animal for these experiments, it could not be used for studying gastric motility or the absorption coefficient in the unæsthetized state.

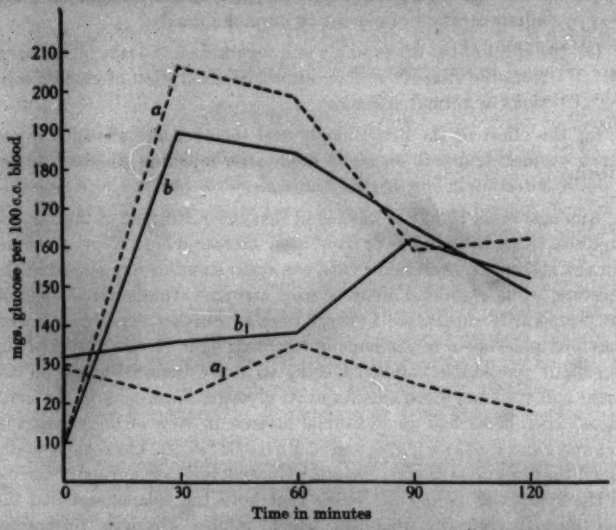

Fig. 1. Effect of ergotoxin and atropine on blood sugar after giving glucose per os. Curves *a* and a_1 from same rabbit; *a*, glucose only; a_1 glucose + drugs. Curves *b* and b_1 from same rabbit; *b*, glucose only; b_1, glucose + drugs. Ergotoxin, 2 mg. subcutaneously 30 min. before and at zero time. Atropine, 5 mg. subcutaneously 15 min. before, at zero time and at 30, 60 and 90 min. after zero time.

Relation of gastric motility to suppression of hyperglycæmia by ergotoxin and atropine.

For this study rats were used. They were fasted for 24 hours and then fed 3 c.c. 0·75 *M* glucose at 40° C. by stomach tube. After 30 min. they were killed, and the residual sugar estimated separately in the stomach and intestine, so that it was possible to determine the absorption

coefficient and also to obtain a measure of the rate of emptying of the stomach. The drugs were injected subcutaneously, as indicated in Table I, which shows the mean results. Two sets of control experiments were done; into one lot of animals a volume of distilled water equal to that of the drug solutions was injected, while another lot received no injection. These animals will be referred to as "injected" and "non-injected" controls respectively. In this way the influence of the emotional disturbance and the pain caused by the injection were controlled. Some experiments were also done to test whether pilocarpine had any influence on absorption. These proved to be of more than general interest, and so are included in the Table. All the absorption coefficients reported in the paper have been tested statistically and the inferences drawn are based on the results of these tests.

The mean A.C. in the non-injected controls was 0·213, which is distinctly higher than that found by Magee and Reid [1931], 0·167, or by Magee and Sen [1932], 0·174. This improvement is doubtless due to the fact that the rats have become progressively tamer as a result of recent reorganization of the rat colony. At present the rats rarely exhibit any sign of emotion on being returned to their cages after being artificially fed. This A.C. then, when compared with the previous ones, indicates that emotion can play a significant rôle in absorption. The value for the injected controls in the present experiments is in accord with this inference. This A.C. was 0·136, *i.e.* a decrease of 36 p.c. compared with that for the non-injected controls. The fall can only have been due to the excitement and pain caused by the injection, as there is no reason for attributing it to the water injected *per se*. The immediate cause was delay in discharge of the solution from the stomach, because the residual sugar in the stomach amounted to 0·240 g. in the injected, and only to 0·175 in the non-injected controls. As can be seen, the rats used in these experiments were selected so as to be of approximately the same weight. There is no general law stating the relationship which, presumably, exists between body weight and the rate of discharge from the stomach of a given volume of a solution of physiological concentration, as 0·75 *M* glucose has been shown to be. In the absence of such a law, comparisons of the rate of gastric discharge could only be made on the basis of equality of body weight. It therefore seems justifiable to infer that the emotional disturbance and pain caused by subcutaneous injections delay the emptying of the stomach. Consequently, the data for the water-injected controls were taken as the standards in order to assess the effect of the drugs.

TABLE I. Effect of drugs on absorption of glucose by normal rats. Average results.

No. of rats	Wt. g.	Injection	Glucose (in g.) remaining in Stomach	Range	Intestine	Range	Absorption coefficient (A.C.)	Standard error (S.E.) of mean A.C.
19	201	Nil	0·175	0·078–0·259	0·038	0·013–0·122	0·213	0·009
20	226	Water	0·240	0·149–0·301	0·025	0·009–0·044	0·136	0·002
10	229	Atropine	0·290	0·217–0·336	0·033	0·013–0·073	0·051	0·009
15	183	Ergotoxin	0·233	0·165–0·309	0·044	0·011–0·101	0·126	0·012
7	205	Ergotoxin + atropine	0·275	0·232–0·336	0·046	0·021–0·072	0·077	0·012
12	204	Pilocarpine	0·149	0·062–0·270	0·109	0·022–0·211	0·147	0·005

Rats previously fasted for 24 hours. 3 c.c. 0·75 *M* glucose at 40° C. given by stomach tube. Period of absorption 30 min. Drugs given subcutaneously: atropine, 0·25–0·5 mg. 5 min.; ergotoxin (B.W.), 0·65 mg. 30 min.; pilocarpine, 0·5 mg. 30 min., before feeding glucose.

TABLE II. Effects of drugs and of vagotomy and splanchnotomy on alimentary hyperglycæmia and on absorption of glucose injected into the small intestine of amytalized rabbits. Average results.

No. of rabbits	Blood sugar mg./100 c.c. Before anæsthesia	60 min. after operation initial value	Range	Treatment	Increase in blood sugar p.c. over initial value at min. after glucose 20 min.	Range	40 min.	Range	60 min.	Range	Absorption coefficient (A.C.)	Standard error
14	110	133	100–165	Nil	36	15–65	58	20–80	57	41–110	0·041	0·005
10	126	145	103–202	Ergotoxin + atropine	9	−10–36	35	13–70	54	15–88	0·065	0·004
17	123	143	93–206	Ergotoxin	26	−1–57	53	24–104	72	29–147	0·070	0·006
8	118	145	120–161	Atropine	54	14–85	85	49–126	104	68–126	0·071	0·005
10	120	141	122–165	Vagotomy	37	14–81	71	31–135	94	40–143	0·069	0·006
6	120	142	119–177	Splanchnotomy	32	16–47	72	36–112	120	90–141	0·079	0·002

Absorption period 1 hour. Rabbits previously fasted 18 hours. 30 or 40 c.c. 0·75 *M* glucose at 40° C. injected into intestine 1 hour after completion of acute operation. Drugs given subcutaneously: ergotoxin, 2 mg. 30 min. before and 2 mg. with glucose; atropine, 5 mg. 15 min. before, 5 mg. with glucose and two similar doses at 20 min. intervals after glucose. Vagotomy was acute. Splanchnotomy was done 10 days previously.

Ergotoxin plus atropine depressed the A.C. by 43 p.c., from 0·136 to 0·077, apparently by delaying gastric discharge. It should be stated that we do not think very much importance can be attached to the amount of residual sugar in the intestine unless, as in the pilocarpine series, this is relatively very great. In earlier work [Macleod, Magee and Purves, 1930] large variations in intestinal sugar were found in individual animals and it would, therefore, be wrong, in our opinion, to infer, from small differences in the amount of sugar left in the intestine, corresponding variations in the rate of absorption. In the pilocarpine-injected animals, however, the amount of sugar retained in the intestine was so much greater than in any of the other series of animals that some significance must be attached to it. This point will be discussed later.

It has been noted that ergotoxin plus atropine decreased the A.C. by slowing gastric discharge, and it is clear from the effects of injecting atropine and ergotoxin separately, that it was the former and not ergotoxin which was responsible for this inhibition. Thus, ergotoxin had no significant influence on either the A.C. or discharge from the pylorus, whereas atropine lowered both more than the two drugs together. It would seem, then, that ergotoxin counteracted, to some extent, the inhibitory action of atropine on the rate of emptying of the stomach.

The blood sugar of the ergotoxin-atropine series was determined at the end of the experiment. The mean value was 133 mg./100 c.c. which was almost identical with that for the non-injected controls, 134 mg. This result seemed to indicate that, although the drugs lowered the rate of absorption in rats, they did not as in rabbits depress alimentary hyperglycæmia. It was suspected that the cause of the discrepancy might be related to the more rapid metabolic rate in the rat. Further experiments were therefore done in which an absorption period of 15 min. was allowed, the technique otherwise being the same as in the previous experiment except that the drugs were injected 30 min. before feeding, in order to give them time to act. Earlier experience had shown that the effect of atropine had not worn off after this time. The average results are shown in Table III.

TABLE III. Effect of ergotoxin and atropine on the A.C. and blood sugar after a 15 min. absorption period.

No. of rats	Injection	Wt. g.	Glucose in stomach g.	Glucose in intestine g.	A.C.	S.E.	Blood sugar mg./100 c.c.	Range
7	Water	230	0·292	0·030	0·147	0·009	161	125–200
7	Drugs	217	0·297	0·040	0·170	0·018	149	120–188

A.C. = absorption coefficient; S.E. = standard error of mean.

The mean blood sugar value in the drugged rats was only 12 mg. less than that in the controls. This difference is too small to be of any importance.

The A.C. in the water-injected controls, 0·147, was slightly, but not significantly, higher than that in the similar controls, 0·136, shown in Table I. This result supports Cori's [1925] contention that glucose is absorbed at a constant rate. The A.C. of the drugged animals was very variable as the high standard error of the mean indicates. The average A.C., 0·170, however, showed a possibly significant increase over the control average, 0·147. In other words the drugs definitely did not depress the A.C. when the absorption period was 15 min., whereas they had this effect when a 30 min. period was allowed. This apparent contradiction must depend on some other factor concerned in the emptying mechanism of the stomach. The intra-gastric pressure appears to be the most important in this connection. It is conceivable that the initial rise in intragastric pressure, immediately after administering 3 c.c. of fluid, would be sufficient partially to neutralize for a while the inhibitory action of atropine.

The A.C. in the pilocarpine-injected rats was 0·147, which shows a probably significant increase of 8 p.c. over the control value, 0·136 (Table I). The improvement in absorption was very likely brought about indirectly by increasing the rate of discharge from the stomach rather than by stimulating the absorptive process itself. In fact, the latter was most probably slightly depressed. Thus, less residual sugar, 0·149 g., was found in the stomachs of the pilocarpine-injected rats than in the non-injected controls, 0·175 g. By comparing these figures with that for the injected controls, 0·240 g., it becomes apparent that the depressing effect of the injection *per se* on gastric motility was more than off-set by the stimulating action of pilocarpine. In spite of this the A.C. 0·147 was lower than in the non-injected controls, 0·213. The reason for the difference can be seen by comparing the amounts of sugar left in the intestine in the respective series, 0·109 g. in the pilocarpine series and 0·038 g. in the controls. These findings indicate that pilocarpine had a depressing influence on the absorptive process itself.

The foregoing results are summarized here for convenience. After orally given glucose, ergotoxin plus atropine suppress alimentary hyperglycæmia in the rabbit, their effect on the rat being probably transitory and less marked. The suppression is partly due to delay in discharge of the sugar solution from the stomach, in causing which atropine plays a predominant rôle.

Effect of ergotoxin, atropine, vagotomy and splanchnotomy on absorption.

In these experiments the sugar solution was injected directly into the intestine, so that the motility of the pyloric sphincter was ruled out as a complicating factor. An attempt was made to dispense with anæsthetics, by using rabbits in which a loop of the lower ileum had been exteriorized in a preliminary operation. The sugar was injected oralwards into the loop. As the results were very inconstant, the procedure was abandoned and rabbits anæsthetized with amytal were employed instead. This drug, which has no appreciable effect on the blood sugar, was given intravenously in the dosage used by Donhoffer and Macleod [1932]. The animals had previously been fasted for 18 hours. Before giving the amytal a sample of blood was drawn from the ear for estimating the fasting blood sugar. Subsequent samples were taken from the carotid. The abdomen was opened along the linea alba and the pylorus ligated, with thick strands of woollen yarn, so as to minimize damage to any of the structures in the wall. Interference with the nerves going to the intestine had to be avoided and the cannula, accordingly, was tied into the proximal end of the lower ileum. An hour after completion of the operation 30 or 40 c.c., depending on the size of the animal, of 0·75 *M* glucose at 40° were injected through the cannula and the rubber tubing attached to it was then clipped. Preliminary experiments showed that when such volumes were used the solution always reached the duodenum. The dosage and the times of injection of the drugs and the times at which blood samples were drawn are stated in the legend of Table II in which the average results are given. The blood sugar generally kept steady during the hour before the sugar was injected, but at a level about 45 mg. above the pre-anæsthesia value. If the blood sugar showed a marked tendency to rise during this hour, the animal was rejected. As the following average results from five blank experiments show, there was no tendency for the blood sugar to rise significantly during the course of the experiment.

	Blood sugar mg./100 c.c.			
	Min. after operation			
Before anæsthesia	30	60	90	120
126	138	137	146	146

In Table II the initial value means the one determined immediately before injecting the glucose; it was taken as the basis for calculating the

percentage increase in blood sugar. The blood-pressure was only taken in those cases where there was evidence of failing circulation, and if it at any time fell below 40 mm. the experiment was abandoned. It was only in the case of the ergotoxin-injected animals that the circulation and respiration tended to fail, and very often the blood-pressure could be markedly improved by the use of the respiratory pump. Vagotomy was performed as part of the acute experiment, the œsophagus and any stray branches of the nerves being severed just below the diaphragm. Splanchnotomy had to be performed in a preliminary operation, because, when the nerves were sectioned as part of the experiment, rapid collapse of the blood-pressure almost invariably occurred. In thin rabbits the splanchnics were readily found, and about 0·5 cm. of each nerve was excised. The left splanchnics run fairly close to the abdominal aorta, and the right runs obliquely downwards and inwards to join the adrenal near its outer border. Recovery from the operation was usually rapid and deaths were few. About 10 days after the operation the animals were in good enough condition to withstand the acute experiment, the blood-pressure being maintained as well as in animals with intact splanchnics. Acute vagotomy was not, however, well borne by previously splanchnotomized animals. Of six experiments performed, all ended in failure owing to collapse of the blood-pressure. The attempt to determine the combined effect of section of the vagi and splanchnics was therefore unsuccessful.

Results.

The mean values for the A.C. show that each of the factors definitely stimulated the rate of absorption as compared with the control value, 0·041; ergotoxin and atropine together by 58·5 p.c.; ergotoxin alone by 70·4 p.c.; atropine alone by 73·2 p.c.; vagotomy by 68·3 p.c., and splanchnotomy by 92·5 p.c. It follows, therefore, that the stimulation of absorption brought about by ergotoxin and atropine when injected separately and together was brought about in some way by their paralysing action on the autonomic nerves. As to the precise manner in which absorption is promoted by blocking the vagus and splanchnics we have no opinion to offer. Nor would we go so far as to say that these nerves normally carry impulses which inhibit absorption, because we have not tested the effects on absorption of stimulating them. The slight depressing effect of pilocarpine on absorption (Table I) would, however, suggest that the vagus conducts such impulses. The possibility of the occurrence of diffusion through the gut wall into the peritoneal cavity was considered, but no evidence of loss of glucose in this way was ever found. Further-

more, Dobyns and Dragstedt [1933] have recently shown that intraperitoneal diffusion will only take place through devitalized intestine, a condition we never detected.

The blood sugar findings, owing to the variations they exhibited, could not be treated statistically, and the influence of the several factors cannot therefore be regarded with the same degree of certainty as the absorption results. While the latter show that each factor had a definite effect on absorption, the blood sugar results are merely suggestive. Variations occurred, not only after injection of glucose into the intestine, but also before, and were due presumably in the latter case to differences in the response of the animals to amytal or to unavoidable technical irregularities. However, the presence of an initially high or low blood sugar did not appear to affect the curve materially after the sugar was given, as the following data illustrate. They are taken from animals showing the highest, lowest and approximately the mean initial values in the control series.

Initial blood sugar mg./100 c.c.	Percentage increase in blood sugar over initial value at various times after giving glucose		
	20 min.	40 min.	60 min.
165	31	80	80
100	39	50	41
134	26	43	55

Furthermore, four blank experiments were carried out on rabbits to control the influence of the drugs, saline instead of glucose being injected into the intestine. It was found that ergotoxin and atropine, injected at the time and in the dosage stated in Table II, had no influence on the blood sugar level. It appeared reasonable, then, to regard the mean percentage increases as affording an indication of the trend of the blood sugar in each series of experiments. The results (Table II) demonstrate that the increases in the rate of absorption brought about by atropine, vagotomy and splanchnotomy were accompanied by increases in the degree of hyperglycæmia. Indeed the percentage increase in blood sugar at the 60 min. interval shows a close correspondence with the A.C. The increased hyperglycæmia caused by these factors is therefore easily understandable. On the other hand, the improvement in absorption caused by ergotoxin plus atropine or ergotoxin alone was not reflected in the blood sugar, except to a slight extent in the ergotoxin series at the 60 min. interval. This lack of correspondence was clearly due to ergotoxin and not to atropine, although the latter evidently prolonged the transient depressing effect of ergotoxin on hyperglycæmia, which, as the

splanchnotomy series shows, was not due to interruption in sympathetic impulses but rather to some other action of the drug. It is well known that ergotoxin depresses the blood-pressure, an effect which was frequently observed in these experiments; but the precise manner in which this is brought about does not appear to be clearly established. It is, however, conceivable that wide dilation of the hepatic capillaries would enable the liver cells to absorb more glucose than normal from the portal blood and synthesize it to glycogen, so that the development of hyperglycæmia would be delayed temporarily. Thus, in all experiments, except the ergotoxin-atropine and ergotoxin series, increased absorption was accompanied by corresponding increase in the degree of hyperglycæmia; and, as has been suggested, it is probable that these exceptional results are attributable to a property of ergotoxin different from, or secondary to, mere inhibition of sympathetic impulses, and furthermore that this (hypothetical) action is reinforced by atropine.

Recently Niccolini [1933] has obtained results which might be taken to be contradictory to ours. He found that the hyperglycæmic response to glucose ingestion was less in vagotomized than in normal rabbits. His data are not, however, comparable with ours because he administered the glucose into the stomach by catheter.

The site of action of atropine.

Experiments were then conducted in an attempt to locate the site of action of the drugs—whether blocking of the nervous impulses apparently concerned with absorption occurred in the nerve fibres, or whether the action was a local one on the absorbing epithelium. Surviving intestine of rabbits was employed, the method being the same as in previous work on absorption. In each series of experiments the rate of diffusion of glucose from the lumen of intestinal segments of equal length into oxygenated Tyrode's solution at 40° was compared with that into Tyrode containing 10 mg. atropine per 50 c.c. As the examples of results cited below demonstrate, atropine had no definite influence on diffusion of glucose through the wall of surviving intestine.

TABLE IV. Diffusion of glucose through surviving intestine.

		30 min.	60 min.	90 min.	120 min.	
I.	Outer fluid					
	Tyrode	27	56	113	175	mg. sugar/100 c.c.
	Tyrode + atropine	34	88	150	195	
II.	Tyrode	21	38	67	99	mg. sugar/100 c.c.
	Tyrode + atropine	14	33	52	77	

In each experiment 4 c.c. 0·75 *M* glucose were inserted into neighbouring loops of equal length.

No evidence, therefore, was obtained in this way as to any local action of the drug on structures in the intestinal wall; these structures were, however, moribund and not comparable with those in the living animal. As there is no reason for believing that, in regard to absorption, the locus of action of ergotoxin differed from that of atropine, similar experiments with the former drug were considered unnecessary.

Effect of ergotoxin and atropine on liver glycogen.

It is clear that the foregoing results are contrary to, rather than in accordance with, the theories of Pollack and of Hetenyi and Pogány. But, as ergotoxin plus atropine invariably suppressed hyperglycæmia, even when the glucose solution was injected into the intestine, it was necessary to determine whether corresponding changes were to be found in the liver glycogen. These experiments would also, it was hoped, provide an additional test of the above theories. Accordingly, in some of the experiments reported in Table II, glycogen was estimated in pieces of liver (about 1 g.) removed (*a*) immediately after opening the abdomen, and (*b*) at the conclusion of the experiment. In seven control and three animals treated with ergotoxin-atropine the following data were obtained:

	Liver glycogen p.c.							
60 min. before glucose	1·349	1·039	0·511	0·718	3·757	2·542	2·616	Controls
60 „ after „	0·640	0·115	1·142	0·211	3·020	2·114	2·207	
60 „ before „	1·491	2·926	0·375	Ergotoxin and atropine injected				
60 „ after „	1·067	3·052	1·757					

It is strange that, although each rabbit had absorbed during the experiment 1 g. or more of glucose, the glycogen content of the liver fell in six out of the seven control experiments. The unexpected nature of this result as well as the extremely wide variations in the initial value indicated that the procedure was unreliable. Furthermore, the results of the three estimations carried out after injection of ergotoxin-atropine were just as inconstant as the control ones. This line of enquiry was consequently abandoned, and an attempt was made to throw some light on the problem by using the decerebrate rat preparation [Bell, Horne and Magee, 1933]. We are indebted to Miss M. Dennehy, M.Sc., for her co-operation in this work.

Experiments on decerebrate rats.

Rats after 18 hours' fast were decerebrated by the method described by Bell in the above-cited paper, ether anæsthesia being employed. A cannula was fixed in the ileum and the pylorus tied as in the rabbits.

An hour was allowed to elapse to permit the animal to recover from the shock of the operation and 3 c.c. 0·75 *M* glucose were put into the intestine by the cannula. Absorption was allowed to proceed for an hour. The rat was then killed and pieces of liver excised for estimation of glycogen. Atropine was the only drug tested. The results are shown in Table V.

TABLE V. Decerebrated rats. Influence of atropine on liver glycogen, A.C. and blood sugar Glucose, 3 c.c. of 0·75 *M*, injected into intestine.

Series	No. of rats	Treatment	A.C.	S.E.	Blood sugar mg/100 c.c.	Range	Liver glycogen p.c.	Range
1	8	Ether only, not decerebrated	—	—	115	84–134	0·09	0·04–0·22
2	6	Decerebrated and operated	—	—	140	113–155	0·12	0·05–0·27
3	10	Glucose injected into intestine	0·122	0·010	302	146–410	0·06	0·02–0·14
4	11	Glucose and atropine (1·5 or 2 mg.) injected	0·105	0·008	329	232–420	0·16	0·02–0·56

Decerebration was done under ether, then the abdominal operation was completed. An hour then elapsed before glucose was injected and an hour later the experiment was ended. Atropine was given in three or four doses of 0·5 mg. each before and after giving glucose. Blank experiments as indicated were carried out on series (1) and (2). Series (1) were killed 60 min. after a 5 min. period of ether anæsthesia, and series (2) 60 min. after the operation.

The most striking thing about these results is that, although the sugar-fed animals absorbed during the hour 0·1 g. or more of glucose, the glycogen content of the liver was not increased. Cori [1926], on the other hand, found that the glycogen content of the liver in intact rats was increased by 0·38 g. p.c. during an absorption period of an hour. The fact that his average A.C. was 0·183 as compared with our 0·122 cannot be regarded as the reason for the great difference between his results and ours. It would, therefore, seem that the failure to form glycogen in our experiments was due to the operative procedure employed; but it is impossible to say whether decerebration was any more to blame than the abdominal operation. The values in the non-sugar-fed controls were only slightly more variable (0·05 to 0·27 p.c.) than those obtained by Catron and Lewis [1929] in intact rats fasted 24 hours (0·05–0·23 p.c.), thus suggesting that it was not the pre-formed glycogen which was affected by the operation, but rather the ability of the liver to synthesize glycogen from glucose absorbed into the portal vein. The blood sugar results show that the mean value in the atropinized rats was 27 mg. higher at the end of the experiment than in the sugar-fed controls. The variations in the values of the two series were, however, so wide that no importance can be attached to such a small difference.

Atropine failed to have an effect on the liver glycogen, and also lowered the A.C., statistical analysis indicating that the difference between 0·122 and 0·105 is possibly significant. In respect to the A.C. the results obtained in the rats disagreed with those in rabbits under amytal. The cause of this discrepancy was investigated by determining the influence of acute vagotomy on decerebrated and on intact rats under amytal. The average results are shown in Table VI.

TABLE VI. Effect of vagotomy on absorption of glucose, 3 c.c. 0·75 *M*, injected into intestine.

	Controls			Vagotomized			
	No. of rats	A.C.	S.E.	No. of rats	A.C.	S.E.	Increase p.c. over controls
Decerebrated	9	0·122	0·010	10	0·151	0·008	24
Under amytal	9	0·166	0·010	6	0·182	0·017	10

The increase of 24 p.c. caused by vagotomy in the decerebrated rat was found to be significant and the increase of 10 p.c. in animals under amytal possibly significant. It is clear, then, that the cutting-off of vagal impulses increased the rate of absorption of glucose. The finding is of still greater importance in that it is not conditioned by possible effects of anæsthesia as in the case of rabbits under amytal. Indeed, the effect of vagotomy was markedly lessened by amytal anæsthesia. The failure of atropine to increase the A.C. in rats therefore appeared to be due to some other than vago-inhibitory effects of the drug. The finding of Donhoffer and Macleod that the suppression of hyperglycæmia by atropine depends on the nutritive state of the animal suggested that its influence on absorption might be similarly conditioned. The A.C. was therefore determined in non-fasted decerebrated rats, seven of which received 6–10 mg. atropine, and five were controls. The A.C. was respectively 0·184 (S.E. 0·013) and 0·153 (S.E. 0·011), so that atropine raised the rate of absorption by 20 p.c., an increase which was found to be probably significant. It will be noted that the control A.C. in the above series is higher than that for rats fasted 24 hours, 0·122 (Table VI). This is in accord with previous experience, that the rate of absorption of glucose varies inversely as the length of the preliminary fast, an observation made first by Cori [1927]. The most probable cause, as has been suggested [Magee, 1930], is the progressive degeneration which fasting produces in the intestinal epithelium [Sun, 1927]. It is not clear why atropine should stimulate absorption in the fed but not in the fasted animal. It is, however, possible that the factor involved is the condition

of the intestinal mucosa which, when adversely affected by fasting, may be less susceptible to stimulation by atropine than when healthy. The fact that the drug had no effect on diffusion through the isolated intestine (Table IV) is in harmony with such a view, because the tissues in this preparation are already in a dying state. The effect of smaller doses of atropine was then tried, in the hope of throwing further light on the matter. In seven experiments on decerebrated rats fasted for 24 hours and injected with 0·5–0·25 mg. of atropine, the A.C. varied from 0·095 to 0·220. The very wide variation in the results showed that the problem was not to be easily solved, and work on it was therefore discontinued.

Effect of ergotoxin and atropine on the portal blood sugar.

It has been shown [Magee and Reid, 1931] that during absorption of glucose the curve of portal blood sugar runs parallel with, but at a level about 20 mg. higher than, that of the systemic blood (inferior vena cava). Experiments were then begun to determine whether this relationship was altered by ergotoxin and atropine. It is obvious that, if the drugs suppressed alimentary hyperglycæmia in the systemic blood but not in the portal blood, the disappearance of sugar could be assumed to take place in the liver. Rabbits and cats under amytal were used for the experiments, blood samples being taken from the carotid artery and the portal vein at intervals before and after injecting glucose into the intestine. The results were extremely variable and the respective curves behaved in so irregular a fashion that no definite relationship between them could be discerned.

No additional light, then, was thrown on the *modus operandi* of the drugs in lowering alimentary hyperglycæmia, so that these experiments and those on liver glycogen failed to accomplish what we hoped they would do. We are, therefore, driven back on the opinion previously expressed, that the suppression of alimentary hyperglycæmia by ergotoxin and atropine is probably attributable to circulatory changes brought about by ergotoxin and intensified by the simultaneous influence of atropine. But obviously, before this opinion could be established as true, the precise action of ergotoxin on the circulation would have to be worked out.

It would be out of place here to cite or discuss the numerous theories and conceptions which bear on the genesis of alimentary hyperglycæmia. Those of Pollack and of Hetenyi and Pogány have been already shown to be out-of-step with the results of the present experiments, and the same may be said of that put forward by Eisner [1926], Allard

[1926] and Wachsmuth [1930]. These observers contended that alimentary hyperglycæmia was due to reflex excitation of hepatic glycogenolysis by the presence of sugar in the intestine; but their view is irreconcilable with the results showing the effects of atropine, vagotomy and splanchnotomy on the rate of absorption and on hyperglycæmia [Table II]. In these experiments, as the amount of glucose injected was constant, the intensity of the (hypothetical) stimulus would also have been constant and yet different degrees of hyperglycæmia resulted, which varied directly as the rate of absorption. This result, on the contrary, confirms those of Magee and Reid [1931] and of Henry, Macdonald and Magee [1933], and it would therefore seem that the overflow hypothesis of Ernst and his colleagues [1931, 1932] is nearer the truth than any of the other theories.

Summary.

Ergotoxin and atropine suppressed hyperglycæmia in normal rabbits after administration of glucose by stomach tube. Experiments on normal rats showed that this action of the drugs was partly due to delayed absorption, and this in turn to inhibition of gastric discharge, in causing which atropine played a dominant rôle. When glucose was injected into the intestine of rabbits under amytal, atropine, splanchnotomy and vagotomy increased the rate of absorption of glucose and the degree of hyperglycæmia. Ergotoxin and ergotoxin plus atropine also stimulated absorption, but temporarily suppressed hyperglycæmia. Atropine had no effect on diffusion of glucose through the wall of surviving intestine. The promotion of absorption by the drugs and of hyperglycæmia by atropine when given separately was therefore probably due entirely to inhibition of impulses passing down the autonomic nerves. The precise rôle played by these nerves in absorption is not clear from the experiments. The inhibition of alimentary hyperglycæmia by ergotoxin is probably attributable to circulatory changes.

In decerebrated rats, vagotomy increased the rate of absorption, whereas atropine had this effect in non-fasted animals only and depressed absorption in fasted animals.

It is considered that these results are more in favour of the view that alimentary hyperglycæmia is due to overflow of absorbed glucose through the liver than of the rival theories which have been discussed.

Attempts, which were unsuccessful, were made to elucidate the mode of action of the drugs by studies of the liver glycogen in rabbits under

amytal and in decerebrated rats and also by comparisons of the portal and systemic blood sugar during absorption of glucose in rabbits and cats under amytal.

Subcutaneous injections *per se* depressed absorption of orally given glucose by inhibiting gastric discharge.

Pilocarpine increased gastric discharge but slightly depressed absorption.

We wish to express our thanks to the Eli Lilly Co., Indianopolis, U.S.A., for the supply of amytal used in the research, and to Dr P. White, without whose help the statistical analyses could not have been so satisfactorily carried out.

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EXAMINATION OF THE PULMONARY CIRCULATION WITH THE MICROSCOPE.

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The flow of blood in the lung vessels has been observed with the microscope in the entire animal by various workers. Hall [1925] using cats or rabbits anæsthetized with chloretone or urethane, or pithed, studied the circulation at the edge of the lung by transillumination. The chest was opened and the lung suspended by clips to allow the edge to be transilluminated. He noted a distinct slowing and backward movement of the blood stream between the heart beats on stimulation of the vagi and slowing of the heart. He also claims to have seen constriction in the smaller vessels on injection of adrenaline. Wearn, Barr and German [1926] and Wearn, Ernstene and Bromer [1928] observing in cats, under amytal anæsthesia, the vessels at the edge of the lung through a window cut in the thorax with transillumination through a window in the diaphragm from a source of light in the abdomen, noted changes in the number of capillaries with changes in the systemic blood-pressure. They noted that the pressure on the abdominal aorta caused new capillaries and sometimes arterioles to open up, and injection of adrenaline with a rise in systemic blood-pressure to cause an opening up of capillaries.

Olkon and Joannides [1930] examined the capillaries near the lung surface in the dog, using reflected light with cedar oil or glycerine on the pleura. Ether and scopolamine were used. Using artificial respiration they describe changes in the number of capillaries and the flow of blood in them due to alterations in the intrapulmonary pressure. With distension of the lung to a size greater than the normal the single-celled capillaries became fewer, and there was an increase in the flow of blood in the multicellular capillaries surrounding the alveoli. When the intrapulmonary pressure was reduced, the flow in the larger capillaries assumed a to and fro movement, stopping and moving on again.

The present work was undertaken to determine how far alterations in the total pulmonary blood flow and resistance, caused by injection of drugs or by nerve excitations, influenced the smaller blood vessels at the edge of the lung which could be observed with a microscope. To do this the intact animal was first used and the vessels observed by a modification of Hall's method. Later, however, a series of observations were made on isolated lungs perfused with blood at constant pressure, the inflow and outflow of blood being recorded [Gaddum and Holtz, 1933]. By this means it was hoped to correlate the changes in total flow with that taking place locally in blood vessels in the field of the microscope.

Preliminary experiments were undertaken with a view to determining the type of animal most suited for this particular type of examination and any individual variations that might occur in examination of the lungs in different species. At the same time it was hoped to obtain information of the most suitable and simple technique applicable. The animals used were rabbit, cat and dog.

Rabbit. Using Hall's method it was found that vessels containing circulating blood could be seen at the edges of the lungs, but certain disadvantages of this technique became evident. Owing to the delicate texture of the lungs it was difficult even with the most careful manipulation to fix an area for examination without interfering with the circulation at that particular part, moreover when a satisfactory field was under observation the blood circulating in the vessels tended to develop stasis after a short time. Such stasis was apt to occur in vessels at the edges of the lobes after the chest was opened, and, with positive pressure ventilation, even in lobes that had been free from any form of manipulation right up to the time of examination. The lung tissue was apt to tear at the point of attachment of the clips on the slightest traction, and finally failure of the general circulation was much commoner than with the other species of animal experimented on.

Cat. In these animals the preliminary experiments gave much better results. The translucency of the lungs at their borders was such that the blood could be clearly observed circulating in vessels up to the size of 100 microns and, in a few cases, in larger vessels. The lung tissue, whilst being sufficiently translucent, resisted the effects of exposure, and the necessary lung manipulation did not produce stasis in the vessels of the part under examination.

Dog. Two experiments showed that whilst the lung was well suited to resist exposure and manipulation, a good circulation being maintained in the vessels at the very edge of the lobes, the tissue was relatively

opaque so that the vessels and their contents could only be seen indistinctly. Dog lung and rabbit lung were therefore thought less suitable than cat lung for this type of investigation.

Operative technique.

For examination of the vessels with the lungs in situ.

The cats were anæsthetized with ether and medinal (0·42 g. per kg. body weight) and the chest opened under artificial respiration. Several methods were used for exposing the lungs and these can be classified as follows:

(*a*) Extensive removal of the chest wall, the intercostal vessels being ligated. The edges of the lobe to be examined were supported by rubber covered clips. The lobe being thus suspended at a slightly higher level than the others, light could be transmitted through it from a carbon arc lamp, and the lung examined by a microscope fixed horizontally as in the method used by Hall [1925].

(*b*) Using the same apparatus but with a modified operative technique. It was found that if the lowest intercostal space above the insertion of the diaphragm was opened on each side as far as the xiphisternum, the whole chest wall could be lifted upwards and forwards towards the head, the ribs assuming the more horizontal position of deep inspiration. If the pericardium was slung up behind the xiphisternum the heart was kept well in the chest, whilst the lower borders of the lower lobes could project through the space formed and could be transilluminated and examined. This method involved less shock to the preparation. With both these preparations the lungs were kept distended with a continuous insufflation of oxygen [Meltzer, 1909]. The state of distension of the lungs being adjusted by the resistance of the outflow; active positive pressure respiration was given between the observations to prevent any excess of CO_2.

(*c*) The chest was opened and one side partially removed. The bronchus was tied on the open side without involving the pulmonary vascular supply to the lung, air being kept in the lung to maintain the lobes partially distended. The edges of the lobe could now be examined either with a microscope fixed horizontally, or with their edges over the stage of a vertical microscope by suitable arrangement of the preparation. This was found to be useful in the case of the smaller sized animals. In these cases observations could be made following injection of drugs into the circulation but not following nerve stimulation, as by this

method the nerve supply was included in the ligature. The drugs were injected either into a cannula in the saphenous or jugular veins or directly into the right heart. The results from all these experiments are included in Table I.

TABLE I. Cat, entire animal with open thorax. Vagal stimulation.

	Blood vessel under observation			
Date	Size of vessel in μ	Calibre	Flow	Rate and force of heart beat
24. iv. 31	86	N.C.	Slow and rev.	N.C.
11. v. 31*	25	N.C.	Back flow	Heart stopped
18. v. 31	76	N.C.	Stopped, back and forward with each beat	Slowed
18. v. 31	57	N.C.	,,	,,
18. v. 31	76	N.C.	,,	,,
18. v. 31	38	N.C.	,,	,,
18. v. 31	27	N.C.	Stasis	,,
21. v. 31	57	N.C.	Slow and rev.	,,
17. vi. 31	20	N.C.	Slow and back flow between beats	,,

	Adrenaline.				
			Effect upon		
Date	Dose γ	Size of vessel in μ	Calibre	Flow	Rate and force of heart beat
24. iv. 31	100	86	N.C.	Stop and rev.	? N.C.
18. v. 31	100	76	N.C.	+ pulse	+ force
18. v. 31	100	57	N.C.	+ pulse	+ force
18. v. 31	100	76	N.C.	+ pulse	+ force
18. v. 31	100	38	N.C.	+ pulse	+ force
21. v. 31	100	57	N.C.	+ pulse	+ rate
21. v. 31	100	Cap.	Opening	+ pulse	+ rate
17. vi. 31	100	177	Apparent contraction (177 → 90 μ)		
30. vi. 31	3	60	N.C.	Back flow	Slowing
30. vi. 31	10	60	? dilatation	Stop followed by great increase	
5. iii. 33*	1000	60	N.C.	+ rate	+ rate + force

Cap. = capillary; N.C. = no change; + = increased; − = decreased; Sl. = slight; rev. = reversed; P.A. = pulmonary artery. In every experiment in which the blood inflow increased the outflow also increased; also, with an inflow decrease the outflow always decreased.

* Observation repeated three times.

For examination of the vessels in the isolated perfused lung.

The cat was anæsthetized with ether and bled from the carotid, the blood being defibrinated and placed in a Dale and Schuster [1928] perfusion apparatus. A cannula was placed in the pulmonary artery and another in the left auricle, and the lungs removed from the chest.

The lungs were washed through with defibrinated blood, especial care being taken to exclude any air bubbles. They were then attached to the perfusion pump and perfused with defibrinated blood. The lungs lay on a glass stage covering the microscope stage, and the microscope was

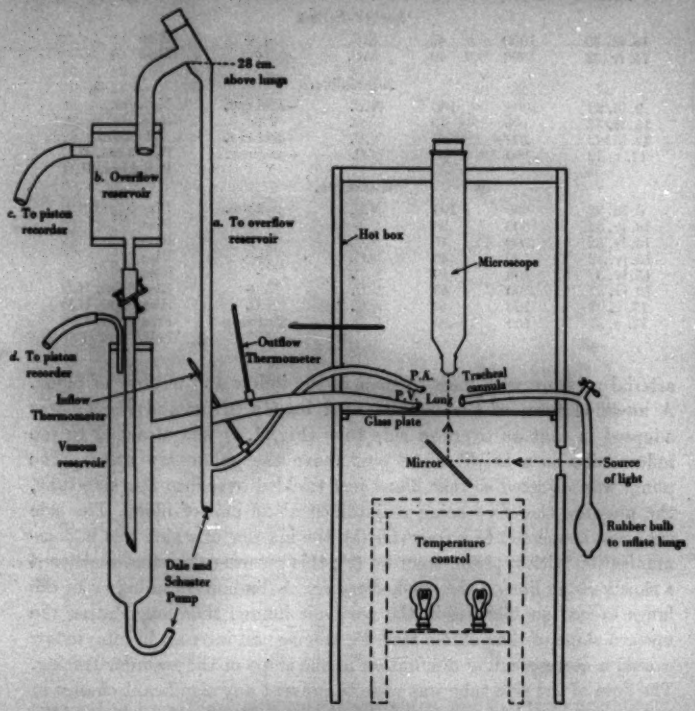

Fig. 1. Diagram of apparatus—see text.

enclosed in a hot box at 38° C. (Fig. 1). By this means the edges of all the lobes could be examined. The lungs themselves were covered in gauze moistened with warm saline, only the edge to be examined being exposed. In one series of experiments the lungs were perfused with a constant blood inflow and the pulmonary arterial pressure recorded (Table II). In a second series, the lungs were perfused at a constant pulmonary

TABLE II. Isolated cat lung perfused at constant blood inflow.

Date	Dose γ	Blood vessel under observation: Size of vessel in μ	Effect upon: Calibre	Effect upon: Flow	P.A. pressure
			Acetyl-choline.		
15. iv. 32	1000	40	N.C.	++	Rise
15. iv. 32	1000	40	N.C.	+	Rise
			Adrenaline.		
9. iii. 32	1000	100	N.C.	−and rev.	N.C.
15. iv. 32	100	40	N.C.	+	Rise
22. iii. 32	200	80	N.C.	−and rev.	Not taken
17. v. 32	100	50	N.C.	−and rev.	Rise 3 cm. H_2O Fall 4 cm. H_2O
			Histamine.		
9. iii. 32	500	100	N.C.	+and rev.	Rise 5 mm. Hg
18. iv. 32	500	30	? +	++	Rise
15. iv. 32	500	40	N.C.	++	Rise
15. iv. 32	100	40	N.C.	+	Rise
15. iv. 32	400	40	N.C.	+	Rise
19. iv. 32	500	40	N.C.	Sl. +	Rise 5 cm. H_2O
17. v. 32	100	40	N.C.	N.C.	Rise 5 cm. H_2O
17. v. 32	100	50	N.C.	−and stop	Rise 1 cm. H_2O Fall 3 cm. H_2O

arterial pressure with measurement of the inflow and outflow of blood. A modified form of the method used by Gaddum and Holtz was adopted in that an overflow side tube (Fig. 1, *a*) was attached to the inflow tube at a height of 28 cm. above the pulmonary artery. The pump was adjusted so that blood just trickled over into this side tube, the pressure therefore being maintained at 28 cm. of blood. The side tube was connected to a reservoir (*b*), the interior of which was in communication with a piston recorder (*c*); this recorder therefore registered a slowly rising line on the smoked paper. A diminution in inflow to the lungs caused an increase in the reservoir inflow, thus augmenting the upward slope of the volume recorder tracing; an increase in lung inflow caused a corresponding diminution in the slope of the recorder tracing. The bore of the side tube was wide to prevent any significant change in pulmonary arterial pressure with an alteration in blood flow. This method has been used by Berry and Daly [1931]. The venous outflow was recorded by connecting a piston recorder (*d*) to the venous reservoir. The total pulmonary changes were recorded simultaneously with observations on the vessel in the field of the microscope. Tracings were taken in every experiment given in Table III and those performed on November 30, 1932, and March 27, 1933, are shown in Figs. 2 and 3. The blood perfused consisted of the blood of the animal from which the lungs were

TABLE III. Isolated cat lung perfused at constant pressure.

Date	Dose γ	Blood vessel under observation: Size of vessel in μ	Effect upon: Calibre	Effect upon: Flow	Total pulmonary outflow
			Acetyl-choline.		
26. vii. 32	4	50	? +	N.C.	N.C.
26. vii. 32	4	Cap.	+	N.C.	+
16. xi. 32	10	62	N.C.	+ +	+
16. xi. 32	50	62	N.C.	+	+
16. xi. 32	100	62	N.C.	+	+
23. xi. 32	100	50	N.C.	+ to −	+ to −
23. xi. 32	100	50	N.C.	Immed. stop and rev.	+
25. xi. 32	5	50	? +	+ +	Sl. +
25. xi. 32	50	50	N.C.	Stop	− −
25. xi. 32	50	50	? −	Slow − rev.	−
26. xi. 32	50	10	N.C.	? +	−
26. xi. 32	5	50	N.C.	+ to −	+ to −
26. xi. 32	50	50	N.C.	N.C.	−
26. xi. 32	50	50	N.C.	+	−
26. xi. 32	50	50	N.C.	+ to −	+ to −
26. xi. 32	1	50	N.C.	+	+ to −
26. xi. 32	5	10	? +	+ to −	Sl. + to −
26. xi. 32	5	50	N.C.	N.C.	+ to −
30. xi. 32	100	50	N.C.	+	+
30. xi. 32	2 mg.	50	N.C.	+	+
30. xi. 32	3 mg.	42	N.C.	+ +	+
30. xi. 32	50	50	N.C.	+	+
30. xi. 32	50	50	N.C.	+	+
30. xi. 32	50	50	N.C.	+	+
30. xi. 32	5	50	? +	+	+
27. iii. 33	100	50	N.C.	N.C.	− −
27. iii. 33	100	50	N.C.	N.C.	−
			Adrenaline.		
26. vii. 32	5	Cap.	N.C.	N.C.	+
23. xi. 32	50	50	N.C.	N.C.	+
23. xi. 32	100	50	N.C.	Sl. −	Sl. −
30. xi. 32	12	50	N.C.	−	−
27. iii. 33	100	50	N.C.	−	N.C.
27. iii. 33	500	50	N.C.	N.C.	Sl. +
			Histamine.		
6. vii. 32	100	80	N.C.	−	−

removed, in some cases with gum saline added, and in a few experiments mixed with blood from another animal. Great variations were shown in different preparations in the time taken before the onset of œdema. In all experiments comparisons were only made in cases where the vessel could be seen clearly and the blood could be observed flowing through it at a good rate. Of the fields examined, any in which the blood stream was slow or stagnant were discarded. There could therefore be no doubt, whatever the state of other areas in the lung, that the one under obser-

vation had an active circulation. The arterioles were recognized by the relative thickness of their walls, together with the observation of the blood flowing from the larger into the smaller vessels.

The examination of most of the fields was made with a magnification of approximately 80 diameters. In certain experiments all lobes were tied off except the one under observation in an attempt to localize the action of the drug.

Fig. 2. (27. iii. 33.) Isolated perfused lungs at constant perfusion pressure. For interpretation of tracings see text. In this figure and in Fig. 3 read from left to right; the upper tracing = overflow reservoir, and lower tracing = venous reservoir. The tracings were taken simultaneously with the observation of the blood vessel under the microscope. At arrow adrenaline (1000γ) was injected. No change is shown on the tracings of the inflow or outflow; at the same time there was very marked slowing of the blood stream in the vessel under observation.

Discussion of results.

On the calibre of the vessels observed.

In twenty experiments with the lungs *in situ*, shown in Table I, where there was evidence of a vascular change taking place in the lung, either as a result of nerve stimulation or injection of a drug, there was no evidence of any alteration in calibre of a vessel observed at the edge of

the lung, except in four cases, two of which were doubtful. In the case where there appeared to be constriction on the injection of adrenaline, the vessel under observation was definitely larger than the average vessel which can be seen microscopically with any degree of clearness near the edge of the cat lung. Evidence is therefore against vessels of a calibre up to 100 μ being affected in calibre to any degree which is visible with the microscope during a vascular change in the lungs. That the change

Fig. 3. Isolated perfused lungs at constant perfusion pressure. (*a*) (30. xi. 33.) At arrow adrenaline (12γ) was injected. Decrease in total pulmonary inflow and outflow with slowing of the blood stream in the vessel under observation. (*b*) (27. iii. 33.) At arrow acetyl-choline (50γ) was injected. Marked decrease in total pulmonary inflow and outflow with no change in flow in the vessel under observation.

takes place in larger and more central vessels which cannot be observed with the microscope is indicated, although not proved, by this absence of change in calibre in these vessels up to 100 μ, and by the calibre alteration taking place in the case of the one larger vessel observed. That a change too small to be detected may be taking place in the vessels under observation is a possibility that has to be considered.

In the isolated perfused lung (Tables II and III) the same results were found. Vessels of approximately the same calibre being chosen in each case. Whereas a marked change is shown to be taking place in the

vascular bed of the lung as indicated by the tracings and the alteration of flow in vessels under observation, there is no change in calibre seen except in five doubtful cases which could not be measured definitely on the micrometer scale. These findings fail to confirm the results of Hall [1925], who found a definite constriction of the vessels at the edge of the lung to take place after injection of adrenaline in rabbits and cats. In the intact preparation in the greater number of cases medinal was used after ether induction, and this might account for a difference found in the results [Daly, 1933], but in the isolated lung preparation no anæsthetic other than ether was used.

On the blood flow in the vessels observed.

In the entire animals marked changes in the rate, and in some cases the direction of blood flow were seen in the vessel under observation, on nerve stimulation or drug injection. On stimulation of the vagus (Table I) a slowing and, in some cases, a definite reversal of flow is found to be taking place in the vessel under observation. In the capillaries a cessation of flow and a breaking-up of the continuity of the cells were observed. This confirms the observations made by Hall [1925].

With adrenaline, changes in flow were also noticeable in the vessel under observation, a decrease or even a complete reversal being seen. Since it was desirable to eliminate the changes due to the cardiac factor the isolated lungs were perfused by the method already described with the results seen in Tables II and III. It will be seen that this affords a means of observing the total change taking place in the pulmonary vascular bed, so that it can be correlated with the changes noted in the vessel under observation. Were the same change to take place equally in all areas of the lung on injection of a drug so as to cause either an increase or a decrease in the blood flow through the lung vessels, the change observed locally by the microscope should agree with the general change recorded by the inflow and outflow of the lung vessels. Thus, on an increase in total inflow and outflow, *i.e.* on increased flow of blood through the pulmonary vascular system, an increase in the rate of flow would be expected in the vessel observed locally, and with a decreased flow a decrease in rate would be expected in the vessel observed. An examination of the results of experiments on the isolated lung summarized in Tables II and III shows that whilst in most cases the local change is similar to the general one, there are several examples of a general alteration in flow with no local one and of a general alteration of flow with the reverse effect taking place locally.

An analysis of the series of readings in Table III where a vascular change has been induced by injection of acetyl-choline, adrenaline or histamine shows the following variations:

Change in total pulmonary circulation—blood flow	Rate of flow in observed vessel	No. of observations recorded
Increase	Increase	17
Increase	No change	4
Increase	Decrease	1
Decrease	Decrease	7
Decrease	No change	4
Decrease	Increase	2

In both the intact and isolated lungs there are examples of complete reversal of flow sometimes lasting for over a minute after the injection, and whilst this reversal of flow usually takes place with a slowing of the

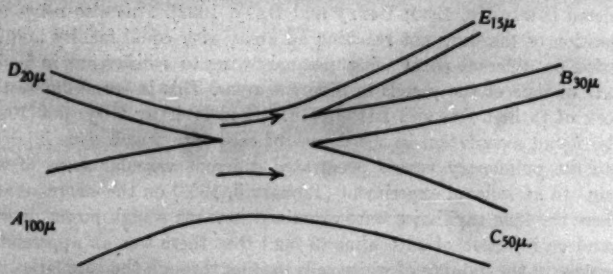

Fig. 4. Diagram of blood vessels seen under the microscope; the arrows indicate the direction of blood flow at the commencement of the observation. The effect of an adrenaline injection on the flow is described in the text.

rate of flow in the vessel, there is at least one observation of a sudden reversal of flow at a marked increased rate following injection of histamine in the intact animal. This anomaly between the local and general flow (Figs. 2, 3) shows that, whatever the alteration in the general vascular flow in the lung may be, it is not referred to all parts of the vascular bed, and in some areas the reverse effect to the general one is taking place. The underlying causes, both physical and physiological, are too numerous for any conclusions to be made at this stage of the work; moreover, the changes in blood flow in the vessels under observation are sometimes extremely complex. An example of this is shown in an experiment (Table II, 17. v. 32), where the following changes were observed taking place. Half a minute after the injection of adrenaline (100γ) the blood-pressure rose 3 cm. (H_2O) followed by a gradual fall, which

continued until 2 min. after the injection, when it had fallen 4 cm. (H_2O) below the initial value. Observations on the blood vessels in the area during this time showed a marked slowing of the stream flowing from *A* to *C*, which had been flowing rapidly at the commencement of the observation, although pulsation was still present in the vessel walls. Complete cessation of flow from *A* to *C* took place 3 min. after the injection, and as soon as this happened the flow, which had continued from *D* to *E*, suddenly reversed its direction so as to flow from *E* to *D*. The blood in *B* gave only oscillatory movements during the whole of the observation. After some time the blood flow recommenced from *A* to *C* and the direction in *D* and *E* returned to normal. These changes are no doubt due to more than one mechanism, but it is worthy of note that the possibility of a Venturi action being responsible has already been considered [Swindle, 1930; Berry and Daly, 1932]. This also raises the question of the drug not reaching all areas with equal facility and its action on different parts being unequal owing to a difference in initial state of tone of the vessels in different areas. This is borne out in the work of Cohnheim and Litten [1875] and later by Toyama [1925] who found a variation in the degree of ease with which dyes injected into the pulmonary vessels penetrated different vascular areas of the lung. In an isolated experiment (January 5, 1933) on the entire animal where the lung capillaries were examined beneath a high power, it was found on injection of adrenaline (2 mg.) that there was an appreciable increase in the number of white cells passing through the capillaries.

The effects on the total pulmonary flow from the drugs used are shown by the tracings of the inflow and outflow in the isolated lungs: those due to adrenaline show dilatation or constriction not related to the dose. Histamine causes constriction, and in any given experiment acetyl choline shows dilatation with small doses but with larger doses constriction alone, or dilatation followed by constriction. This confirms the work of Gaddum and Holtz [1933].

Summary.

The vessels at the edge of the lung were studied beneath the microscope, using transillumination both in the animal with the chest open and the isolated perfused lungs. No appreciable change in calibre of arterioles of a diameter up to 100 μ was observed on injection of adrenaline, acetyl-choline or histamine, or by nerve stimulation, although these procedures frequently brought about an appreciable alteration in the total pulmonary blood inflow and outflow.

A definite alteration in rate, and in some cases in direction of blood flow, was noted and their significance in their correlation with the changes in the total vascular bed is discussed.

I am greatly indebted to Prof. I. de Burgh Daly, who suggested this work, for his helpful criticism and advice.

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612.621

INDUCTION OF OVULATION IN THE UNMATED ŒSTROUS FERRET.

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(*Received August* 3, 1933.)

All early work on the induction of ovulation by means of gonadotropic substances was carried out on animals which ovulate spontaneously, and thus the possibility of examining the precise relationship between time of injection and rupture of the follicle was precluded. Bellerby [1929], however, showed that a single intravenous injection of an acid extract of anterior lobe of pituitary would bring about ovulation in the unmated œstrous rabbit (a species which normally ovulates only after copulation) and that the latent period was about the same as after copulation. This finding fits in well with the fact that the amount of hormone necessary to bring about ovulation is secreted by the pituitary within 1 hour after copulation [Fee and Parkes, 1929]. Hill and Parkes [1930 *a*] confirmed Bellerby's findings by the use of extracts prepared from urine of pregnancy, and further showed that the corpora lutea resulting from ovulation develop normally and are capable of maintaining a pseudo-pregnancy of the usual type. These authors also obtained ovulation in the anœstrous ferret by means of extracts of anterior pituitary and of pregnancy urine and again showed that the corpora lutea formed after induced ovulation are capable of carrying out their normal function [1930 *b*].

In the ferret, the other common animal ovulating only after copulation, the latent period after copulation is about three times that of the rabbit, *i.e.* 30–40 hours [Hammond and Marshall, 1930], and it seemed desirable to extend the work on this species and to investigate the following points:

(1) Whether, in view of the lability of the substance producing ovulation, a single intravenous injection would cause ovulation in the unmated œstrous ferret.

(2) What amount of hormone would be required compared with that necessary to induce ovulation in the unmated œstrous rabbit.

(3) Whether ovulation would follow injection more rapidly than it follows copulation.

The first question was readily settled by a single experiment. The second offered no difficulties other than the use of several animals. The answer to the third was foreshadowed by the findings of Hill and Parkes [1932], who were unable to prevent ovulation in ferrets by hypophysectomy 1 hour 50 min. after the beginning of copulation; the substance producing ovulation had presumably been secreted into the blood within a short time after mating.

Material and technique.

Extract. The anterior pituitary extract used in these experiments was made from ox glands dried by acetone. This material was obtained through the kindness of the British Drug Houses to whom the author expresses his best thanks. The stock was kept in the form of a dry powder from which the various dilutions were made as required. The same sample (Batch 1) was used throughout the work.

Injections. The extract was tested on rabbits before being used for ferrets. This was done by the ordinary method, *i.e.* injection into the marginal ear vein and examination of the ovaries, under anæsthesia, 24 hours later. In ferrets the injection was made into the external saphenous vein. The animals were lightly anæsthetized, the hair on one of the hind legs shaved off and an incision about $\frac{1}{2}$ in. long made through the skin. The vein is readily seen embedded in the subcutaneous tissue. After injection the vein was tied off and the skin wound closed with catgut. Examination of the ovaries of the ferrets usually took place 48 hours after injection, except in those experiments where an attempt was made to obtain "accelerated" ovulation. In these animals one ovary was removed at the required time after injection, and the other left until autopsy.

In each species the approximate amount of hormone required to cause ovulation was first determined and then further tests were carried out near the critical point.

Histology. Owing to the difficulty of diagnosing ruptured follicles in the fresh ovary of the ferret all material was examined histologically in serial sections. In several animals the ruptured follicles seemed atypical, and in these cases final diagnosis was made by examination of the Fallopian tubes for ova.

Experimental results.

Twelve unmated œstrous rabbits and eleven unmated œstrous ferrets were used. Of the twelve rabbits four received 0·5 mg. or more of the extract, and all of these four ovulated; seven received 0·25 mg., and of these only one ovulated; one also had 0·1 mg. without result. The effective dose therefore in the unmated œstrous rabbit is approximately 0·5 mg.

Of the eleven ferrets two received 0·5 mg. and seven others doses larger than this. All of these ovulated except one having 0·5 mg.; one having 0·25 mg., and one 0·1 mg. both failed to react. Approximately the same amount of the extract therefore (0·5 mg.) is required to cause ovulation in the unmated œstrous ferret as in the rabbit in this condition.

Attempts to accelerate ovulation in the unmated œstrous ferret by means of anterior pituitary extract were made with the results shown in Table I.

Table I.

		Result			
No. of animal	Amount of material injected mg.	First ovary removed—time after injection hrs.	Ovulation	Second ovary removed—time after injection hrs.	Ovulation
OF 1	1	12	–	40	+
OF 2	5	12	+	40	+
OF 9	5	18	–	48	+
OF 7	10	24	–	48	+
OF 3	20	19	–	48	+

Discussion.

The similarity of the amounts of anterior pituitary hormone required to cause ovulation in the unmated œstrous ferret and rabbit is significant. Assuming the weight of the rabbit to be four times that of the ferret, the latter requires, weight for weight, about four times as much hormone. The time required for ovulation to take place in the ferret after coitus (30–40 hours), as compared with that in the rabbit (10–12 hours), probably accounts for this large difference. It can readily be supposed that the longer the pituitary hormone has to be in the circulation the greater must be the initial concentration.

"Accelerated" ovulation. The experiments on "accelerated" ovulation (Table I) gave consistently negative results except that OF 2 ovulated 12 hours after injection. This result is anomalous since the same dose, 5 mg., produced no ovulation in another animal 18 hours after injection;

nor did 10 mg. at 24 hours or 20 mg. at 19 hours. In OF 2, however, there is no doubt that ovulation occurred, as the result was verified by the finding of ova in sections of the Fallopian tube.

Summary.

1. The amount of anterior pituitary material required to cause ovulation in the unmated œstrous ferret is approximately the same as in the unmated œstrous rabbit.

2. The ferret thus requires more hormone, weight for weight, than the rabbit, a fact probably accounted for by its longer latent period.

3. Of five attempts to obtain "accelerated" ovulation in the unmated œstrous ferret with anterior pituitary extract only one gave a positive result.

The author expresses his thanks to Dr A. S. Parkes for his kindness and invaluable help.

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612.115.3

THE RELATIONSHIP OF AZO DYES TO THE COAGULATION OF BLOOD.

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(Received September 13, 1933.)

The present work originated with the discovery by Rous, Gilding and Smith [1930] that chicago blue 6B, which they used for determining the gradient of permeability along a capillary, had an anti-coagulant action on whole blood. Parkes and Brambell [1932] used it successfully as an anti-coagulant in perfusion experiments, both in the form of the American preparation, chicago blue 6B, and in the form of the British preparation marketed as chlorazol sky blue FF. They noted only quantitative differences in action.

The mechanism of action of chlorazol sky blue FF as an anti-coagulant was investigated, and a preliminary note published by Huggett and Silman in 1932.

This paper deals only with the relative efficiency of azo dyes as anti-coagulants and the relation between activity and chemical structure. One object in view was to find a dye which is anti-coagulant under all experimental conditions, inexpensive and does not render arterial and venous blood the same uniform "ink" colour as occurs with chlorazol sky blue FF.

Many synthetic water-soluble direct dyes have pharmacological importance and are used in medicine. Such are trypan red [Ehrlich and Shiga, 1904] and trypan blue and afridol violet [Nicolle and Mesnil, 1906]. Subsequently germanin (Bayer 205) appeared. It is, however, not an azo compound. Stüber and Lang [1930] showed that it has anti-coagulant action. In 1920 Harris suggested congo red as suitable for blood-volume determinations by the dye method. All these compounds like chlorazol sky blue FF have anti-coagulant properties.

Methods.

The dyes and other compounds investigated fall into the classes shown in Table I. Most of the dyes used are described in the *Colour Index*, a British official (though not exhaustive) list of synthetic and natural dyes giving their trade names, chemical constitution, mode of preparation and bibliography, and published by the Society of Dyers and Colourists.

In order to make comparable observations a standard technique was adopted. The anti-coagulant effect of each dye was first investigated after mixing the blood with dye *in vitro* and determining the relation between the dose per unit volume of blood and the clotting time. Following this the dye was injected intravenously in doses of 80 and 160 mg. per kg. body weight. The animal was bled at regular intervals and the clotting time obtained. In this way it became possible to obtain the relative anti-coagulant action for the two conditions *in vitro* and *in vivo*. The doses of 80 and 160 mg. per kg. were chosen on the assumption that the blood volume corresponds to 8 p.c. of the body weight, so being equivalent to

Table I.

Class	Colour Index No.	Trade or special name	Molecular weight
I. Disazo dyes:			
(i) Secondary	278	Chlorazol fast red KS	675
(ii) Tertiary	327	Chlorazol fast scarlet 4 BS	813
(iii) From diamines	353	Chlorazol fast pink BKS	862
	—	S.D. 2	862
	—	S.D. 1	664
	370	Congo red	696
	423	Chlorazol brown B	668
	477	Chlorazol blue 3 B (trypan blue)	976
	518	Chlorazol sky blue FF	992
	518	Chlorazol sky blue FF (potassium salt)	1056
	518	Chicago blue 6 B	992
II. Trisazo dyes	581	Chlorazol black E extra	901
III. Tetrakisazo dyes	561	Chlorazol brown LF	1236
	561	Trisulphon brown B	1236
	570	Trisulphon brown G	1266
	577	Chlorazol brown 2 G	1282
	577	Trisulphon brown 2 G	1282
IV. Miscellaneous azo direct dyes	—	Chlorazol fast blue FFKS	
	—	Chlorazol orange brown X	
	—	Diamine fast blue FFB	
V. Triphenylmethane dyes	704	Alkali blue B	573
VI. Miscellaneous	—	Germanin	1472
	—	Novirudin	
	—	Heparin	

The constitution of the miscellaneous substances in Classes IV and VI are either trade secrets or unknown and therefore molecular weights cannot be given.

1 and 2 mg. per 1 c.c. of blood, which the *in vitro* experiments showed to be the important range of activity.

TABLE II. Chemical names and formation of the more important anti-coagulant azo dyes. Structural formulæ obtainable from the *Colour Index*.

Trade name	Colour Index No.	Chemical name
Chlorazol sky blue FF (chicago blue 6B)	518	Tetrazotized dianisidine doubly coupled in alkaline solution with 1-amino-8-naphthol-2 : 4-disulphonic acid.
Trypan red	438	Tetrazotized benzidine-3-sulphonic acid doubly coupled with β-naphthylamine-3 : 6-disulphonic acid.
Trypan blue	477	Tetrazotized tolidine doubly coupled in alkaline solution with 1-amino-8-naphthol-3 : 6-disulphonic acid.
Afridol violet	—	Tetrazotized *p-p'*-diaminodiphenylurea doubly coupled in alkaline solution with 1-amino-8-naphthol-3 : 6-disulphonic acid.
Congo red	370	Tetrazotized benzidine doubly coupled with α-naphthylamine-4-sulphonic acid.
Chlorazol fast pink BKS	353	Tetrazotized *p-p'*-diaminodiphenylurea disulphonic acid doubly coupled in acid solution with 2-amino-8-naphthol-6-sulphonic acid.

(i) *The* in vitro *experiments.*

The rabbits used were anæsthetized with Liquid Dial Co., 0·6 c.c. per kg. A cannula was inserted in the left carotid artery with a bulldog clip upon the artery and a short piece of rubber tubing upon the cannula. Blood was taken by inserting a 1 c.c. pipette into the rubber tubing and allowing the blood to run. The 1 c.c. of blood was then run off into a test-tube containing a known quantity of the dye in solution, which dipped into a water bath kept at 38° C. The clotting time was taken as the time at which the blood set and refused to run on tilting. The cannula and the artery were washed out thoroughly with saline immediately after each bleeding. In this way it was possible to keep the cannula relatively free of thrombokinase and thrombase and at the same time remove blood frequently from the same rabbit.

(ii) *The* in vivo *experiments.*

The weight of dye required was dissolved in 0·9 p.c. sodium chloride and injected into the ear vein of the rabbit. The rabbit was then bled at suitable intervals from the other ear vein into a test-tube, the wound being kept washed with saline and free from adherent clot or thrombogenic enzymes. The clotting time was determined in the water bath in the manner described.

These two techniques described for the *in vitro* and *in vivo* tests are known to give clotting times which are not absolutely maximal, but they

do enable repeated observations to be made on the same rabbit and also allow of comparison between different dyes.

The action of these dyes is largely dependent on the conditions in which they are used. Commercial dyes bought in the open market or direct from manufacturers are far from being pure, containing as much as 70 p.c. of sodium chloride and appreciable quantities of iron, lead, copper, zinc and arsenic. This is because they are manufactured with crude chemicals from crude materials and isolated by salting out with sodium chloride. The product so obtained contains impurities from the chemicals used, together with intermediate products. These impurities are immaterial for dyeing purposes and so are not removed. If the dyes are required chemically pure, it would be necessary to use pure reagents and special plant in their manufacture. This fact of the variations in the purity of different specimens of dyes has not been sufficiently taken into account in previous work on the use of dyes in medicine and, consequently, it is important to emphasize it here.

These considerations have involved the analysis of all the more active dyes for their saline and metallic impurities. We therefore tested the crude samples first and, having determined whether they had any appreciable action *in vitro*, purified them and tested the purified samples *in vitro* and *in vivo*. The effect of purification in the case of two of these dyes is shown in Table III.

TABLE III. Effect of purification on dyes and on coagulation time.

	Dye I			Dye II	
Analyses	Crude Pts/10^6	Purified Sample A Pts/10^6	Purified Sample B Pts/10^6	Crude Pts/10^6	Purified Sample Pts/10^6
As	4	10	6	2·5	5·0
Pb	60	200	120	220	80
Cu	10	60	0	—	Trace
Zn	40	220	120	—	Trace
Fe	60	900	60	500	160
NaCl	70 p.c.	11·7 p.c.	0	30 p.c.	20 p.c.
Coagulation times					
In vitro: 1 mg./c.c. blood	—	4 min.	12 hours	1 hour	24 hours
In vivo: 2 mg./c.c. blood bled after 1 hour	—	6 min.	24 hours	—	24 hours
Same after 3 hours	—	6 min.	2½ hours	—	10 min.

Results.

The action of these substances *in vitro* is best illustrated by Table IV and Fig. 1. Of all the dyes tested in the crude or semi-crude condition, a list of which is given in Table II, only some appeared to merit further

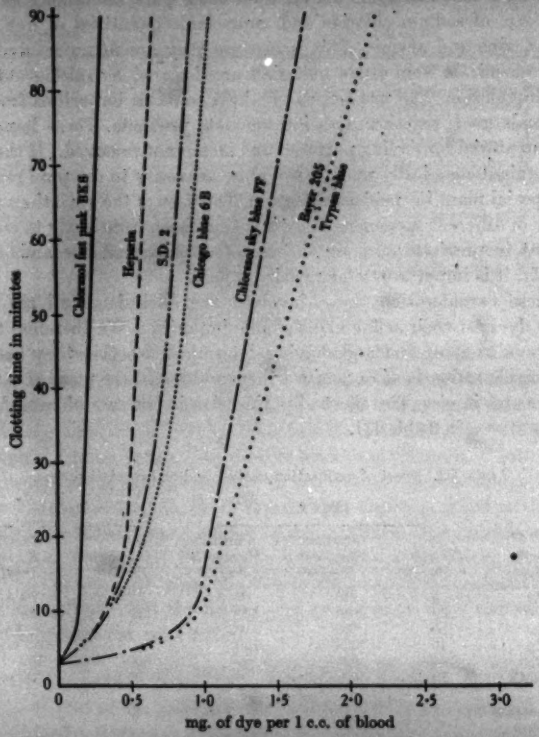

Fig. 1. To illustrate Table IV. Anti-coagulant action of dyes *in vitro*. The effect on the clotting time of mixing *in vitro* definite quantities of the dye with 1 c.c. of blood.

purification, and only those so purified are included in Table IV. Certain other substances are given for contrast, notably heparin and novirudin as well as germanin (Bayer 250) and quinanil. Quinanil is an anil-quinoline derivative used as an antiseptic by intravenous injection

TABLE IV. Relation between anti-coagulant dose (mg./c.c.) and the clotting time *in vitro* (minutes).
(Dyes specially purified.)

Concentration mg./c.c. blood	Heparin	Colour Index No. 518 Sample 1	Sample 2	Sample 3	Sample 4	Colour Index No. 353	No. 561	No. 370	No. 327	Bayer 205	Novirudin	48S	S.D.1	S.D. 2
0·1	—	5	4*	—	5	11	3	—	—	—	3	—	7	—
0·2	5	3	—	2	—	37	—	—	—	—	—	—	11	—
0·25	—	4	—	—	5	110	4	—	2·5	—	—	3	13	—
0·3	—	5	—	—	—	150	6	—	—	—	—	—	14	—
0·4	—	—	—	—	—	12 hours	—	—	—	—	—	—	—	—
0·5	45	7	5	2·5	5	24 hours	6	5	2·75	3	6	18	26	21
0·75	—	9	—	—	16	—	17	—	—	—	—	28	—	42
1·0	4 hours	60	7·5	2·5	24 hours	—	40	100	1·0	11	10	34	40	210
1·25	—	24 hours	28	4	—	—	84	—	—	—	—	—	—	330
1·5	24 hours	—	104	6	—	—	200	—	—	24	30	24 hours	120	24 hours
1·75	—	—	—	—	—	—	—	—	—	—	—	—	12 hours	—
2·0	—	—	24 hours	30	—	—	24 hours	24 hours	40	70	24 hours	—	24 hours	—

Colour Index No. 518 known as chlorazol sky blue FFS or chicago blue 6B (sample 1).
" " " 353 " " chlorazol fast pink BKS.
" " " 561 " " chlorazol brown LF.
" " " 327 " " chlorazol fast scarlet 4BS.
" " " 370 " " congo red.
Colour No. 48S. " " quinanil.
" " S.D.1 and 2 " " special.

* For the figures for sample 2, Colour Index No. 518, we are indebted to Dr A. S. Parkes.

and described by Armitage, Gordon, Cohen, Ellingworth and Dobson [1929].

It will be seen that even in the purified state these dyes vary very much in efficiency. The outstanding ones are Colour Index No. 518, Samples 1 and 4, and Colour Index No. 353. These are all superior to heparin, the latter having been taken as the standard of minimal efficiency which should be required. Colour Index No. 353 is a purified preparation of a dye sold in England under the name of chlorazol fast pink BKS. In regard to the samples of Colour Index No. 518, No. 1 is the original purified chicago blue 6B, for which we are indebted to Prof. H. P. Gilding, Nos. 2 and 3 are samples sold in England under the name of chlorazol sky blue FF as physiologically pure, whilst No. 4 is a sample purified by conversion into the potassium salt. The purified chicago blue 6B in solution has, when compared with the others, a mauve tint suggesting traces of a pink dye as impurity. The other samples were pure blue in colour.

The results obtained on intravenous injection are shown in Table V and Fig. 2. On comparing these figures with those in Table IV and Fig. 1, it will be seen that there is no direct parallel between the action *in vitro* and the action *in vivo*. The efficiency of the dyes *in vivo* may be classified in the following descending order: chicago blue 6B, chlorazol fast pink BKS, heparin, the potassium salt of chlorazol sky blue FF and then the sodium salt of chlorazol sky blue FF. *In vitro*, on the other hand, the descending order is as follows: chlorazol fast pink BKS, heparin, potassium chlorazol sky blue FF, chicago blue 6B and then sodium chlorazol sky blue FF.

This difference in action *in vivo* and *in vitro* is apparently related to the rate at which the dye leaves the blood. In the case of heparin the mode of removal is uncertain. Chlorazol sky blue FF is picked up mainly by the reticulo-endothelial system, to a lesser extent by the tissues in general, and in certain concentrations appears in the urine. It has been shown by Rous, Gilding and Smith [1930] that it diffuses out very slowly from the capillaries into surrounding tissues. It is excreted only to a small extent, if at all, by the kidneys. Animals injected with the dye develop a blue colour in the skin and all other vascular tissues, retaining the colour in these tissues long after it has disappeared from the blood. The suggestion has been made by Dr F. R. Winton that the reason it appears to only a slight extent in the urine is on account of its existing in molecular aggregates of greater total aggregate molecular weight than 70,000, the threshold of molecular size for diffusion of substances from the blood *via* the kidney [Bayliss, Kerridge, Tookey and Russell, 1933].

TABLE V. Anti-coagulant action of dyes on intravenous injection. Duration of fluidity of the blood when removed at stated intervals after injection of the dye. Doses administered: 80 and 160 mg. per kg. body weight. (Dyes specially purified.)

Time from injection of the dye	Heparin mg./kg.		Colour Index No. 518 mg./kg.				Colour Index No. 353 (doses calculated as salt-free dye). mg./kg.				S.D.2 mg./kg.	
			Sample 1	Sample 3		Sample 4						
	80	160	160	80	160	160	32	128	160	256	80	160
5 min.	P	P	P	10 min.	35 min.	P	P	P	P	P	P	P
15 ,,	P	P	P	5 ,,	16 ,,	P	53 min.	3 hours	P	P	25 min.	P
30 ,,	P	P	P	5 ,,	10 ,,	P	36 ,,	1 ,,	P	P	—	P
1 hour	P	P	P	3 ,,	8 ,,	P	10 ,,	33 min.	P	P	20 min.	P
2 ,,	4 hours	P	21 hours	—	5 ,,	P	9 ,,	12 ,,	8 hours	P	17 ,,	P
3 ,,	10 min.	5 hours	5 ,,	—	—	2½ hours	—	—	4 ,,	9 hours	8 ,,	5 hours
4 ,,	3 ,,	2 ,,	3 ,,	—	—	10 min.	—	—	—	8 ,,	—	3½ ,,
5 ,,	—	1 ,,	2 ,,	—	—	11 ,,	—	—	90 min.	4½ ,,	—	3 ,,
6 ,,	—	3 min.	90 min.	—	—	—	—	—	70 ,,	6 ,,	—	2½ ,,
7 ,,	—	—	60 ,,	—	—	—	—	—	—	—	—	2¼ ,,
8 ,,	—	—	—	—	—	—	—	—	—	—	—	—
9 ,,	—	—	10 ,,	—	—	—	—	—	—	—	—	75 min.
10 ,,	—	—	—	—	—	—	—	—	—	75 min.	—	—
11 ,,	—	—	—	—	—	—	—	—	—	29 ,,	—	—
12 ,,	—	—	—	—	—	—	—	—	—	30 ,,	—	—

P signifies permanent fluidity, that is greater than 24–36 hours.

In regard to chlorazol fast pink BKS, this is not only extremely soluble, but it is also extremely diffusible. One result is that in the purification it has been very difficult to obtain a preparation containing less than 20 p.c.

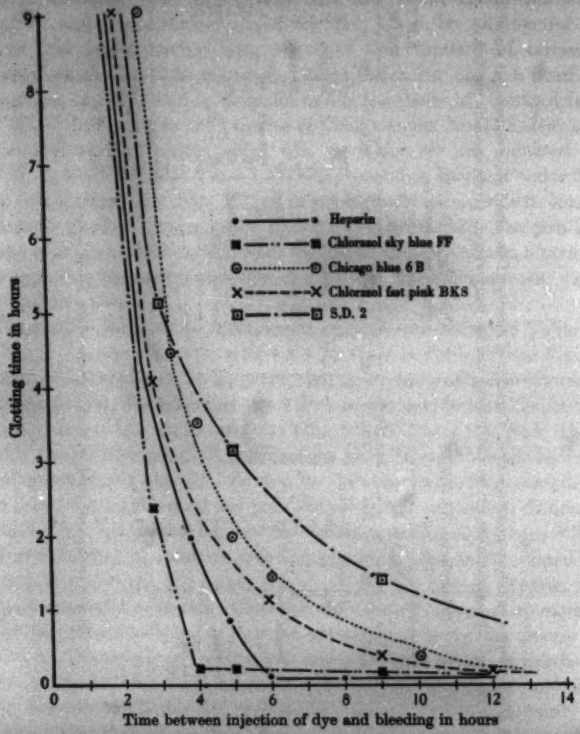

Fig. 2. To illustrate Table V. Anti-coagulant action of dyes *in vivo*. 160 mg. of the dyestuff injected per kg. body weight of animal and the clotting time of the blood determined at regular intervals after injection.

of sodium chloride, unlike the potassium chlorazol sky blue FF, in which case the inorganic salt is reduced by dialysis and other means from 70 p.c. to zero. The second consequence of this high diffusibility is that the dye is excreted more freely by the kidney than is chlorazol sky blue FF. The

figures given in Table IV are recorded for 100 g. of the purified chlorazol fast pink BKS as 80 g. of the salt-free dye, so that in order to give a dose of 160 mg. per kg., 200 mg. of the purified dye (containing 40 mg. of sodium chloride) were injected.

The chlorazol fast pink BKS presents certain advantages, for it is freely soluble, it is the most active compound yet found as an *in vitro* anti-coagulant, it has a low toxicity and it is more active than heparin *in vivo*, although not quite so active as the chicago blue 6B preparation of the dye Colour Index No. 518, when this is given intravenously.

On the basis of these results, therefore, an attempt was made by varying the structural formula of chlorazol fast pink BKS to obtain a pink dye having an activity in the body comparable to the activity of chlorazol fast pink BKS *in vitro* and better than chicago blue 6B.

The first step was to reduce the solubility and diffusibility by omitting the two sulphonic acid groups from the diphenylurea portion of the formula. In this way the dye S.D.1 was obtained. It is much less soluble and diffusible than chlorazol fast pink BKS. It is red in colour and its action *in vitro* is given in Table IV. It will be seen that while it is not so efficient as either chlorazol fast pink BKS, chicago blue 6B or potassium chlorazol sky blue FF (Colour Index No. 518, sample 4), yet it is better than novirudin or the other samples of sodium chlorazol sky blue FF. We find, however, that the dye is too insoluble and toxic for intravenous work.

In the next preparation, S.D.2, the two sulphonic acid groups were introduced into the aminonaphthol portion of the formula. The alteration increased the solubility and diffusibility, but to a lesser extent than in chlorazol fast pink BKS. Thus S.D.2 was made from tetrazotized diaminodiphenylurea doubly coupled in acid solution with 2-amino-8-naphthol-3 : 6 disulphonic acid.

The effect of this is to produce an isomeride of chlorazol fast pink BKS, but the four sulphonic acid groups, being present in the aminonaphthol residues, confer less solubility and diffusibility than when two of them are in the diphenylurea residue, although greater than if only two are present.

The action of S.D.2 *in vitro* is almost as efficient as chicago blue 6B, and it is fourth on our list. *In vivo*, on the other hand, it is superior (in doses of 160 mg. per kg.) to both the chicago blue 6B and the chlorazol fast pink BKS. This prolonged effect is apparently due to a slower removal from the blood.

Toxicity.

The toxic effect upon injection may be apparent within a few hours or may be delayed for some 10 days. In the latter type there are usually symptoms of enteritis and post-mortem serous peritoneal exudate often accompanied by petechial hæmorrhages in the liver, heart, lungs and meninges may be observed.

The minimum lethal dose for these azo dyes is variable and depends upon a number of factors. "Minimum lethal dose" here refers only to the smallest dose which has been found to kill in these experiments and is not used in Trevan's sense of the phrase.

The chief factors which were found to determine the toxicity of these dyes are:

(i) The use of an anæsthetic, which renders all animals more susceptible to the bigger doses of the dye.

(ii) The degree of purification of the dye.

(iii) The solubility of the dye in the solution. The lower the solubility, the greater the toxicity.

Certain of these dyes, notably chlorazol fast scarlet 4BS and S.D.2, tend to precipitate when solutions in water or alkali are kept. This type of dye is the one which causes the delayed type of death.

Discussion.

Anti-coagulants are used in physiology for two main purposes.

(*a*) For certain minor operations requiring fluid blood during periods of 5–10 min. at the site of operation. For this purpose a dose which *in vitro* prolongs the clotting time to 20 min. will prevent coagulation at the site of operation for 5–10 min., since the blood is not at stasis or in contact with the cannula for more than this period.

(*b*) For transfusion over 12–24 hours. A dose which stops clotting *in vitro* for 24 hours is not sufficient, for this case is complicated by the fact that different drugs leave the blood or are destroyed at different rates. Consequently, a dose is required which is sufficiently great when injected to increase and maintain the clotting time of the blood (tested *in vitro*) to greater than normal during a period of 4–6 hours or more. This time will yield adequate fluidity for transfusions, since the blood is not in static contact with foreign materials during the 24 hours. Table VI shows the relationship between these doses and the minimum lethal dose for some of these azo dyes.

In this paper, attention has been directed to the relative efficiency of different azo dyes as anti-coagulants. The mechanism of their action will

TABLE VI. Active and minimum lethal doses of azo dyes.

	Wt. in mg. prolonging clotting time to 20 min. *in vitro*		Duration over which clotting time is increased above normal by intravenous injection of 160 mg./kg. body weight	Minimum lethal dose on intravenous injection of the drug in rabbits
	mg./c.c. blood	mg./kg. body wt.	hours	mg.
Chicago blue 6 B	0·90	70	12	350
Chlorazol sky blue FF	0·80	64	4	160
Chlorazol fast pink BKS	0·20	16	10	300
S.D. 2	0·50	40	15	160
Heparin	0·25	20	5	300

be dealt with in a later publication. Nearly all the dyes examined showed some anti-coagulant powers, though most of them were very feeble and only exhibited the property in very large and toxic doses. The most efficient dyes are disazo direct dyes prepared from tetrazotized diamines coupled with aminonaphthol sulphonic acids (chicago blue 6 B, chlorazol sky blue FF, chlorazol fast pink BKS). Congo red (see Table II) is less effective and more toxic. The most active compounds examined were the isomers chlorazol fast pink BKS and S.D. 2. The essential difference is transference of the two sulphonic acid groups from the diphenylurea residue to the 2-amino-8-naphthol residue. The effect of this is to decrease the anti-coagulant activity *in vitro*, but by reason of a simultaneous decrease in diffusibility the dye remains in the plasma a greater length of time with the consequence that it produces a more prolonged effect *in vivo*. It is therefore very efficient as an intravenous anti-coagulant.

This decrease of diffusibility consequent upon the transference of the sulphonic acid groups is accompanied by a decrease in solubility in saline and in the alkaline plasma, leading therefore to a slow precipitation. Consequently, its increased efficiency is counterbalanced by a toxicity greater than that of chlorazol fast pink BKS.

The factors concerned in toxicity appear to be the solubility of the dyestuff in the plasma and in sodium chloride solutions and the heavy metal salts present in the dye as a result of the commercial method of preparation. It is therefore essential to purify the dyestuff very thoroughly beforehand especially in regard to sodium chloride, the quantity of which must be accurately known.

While no certain light is thrown on the requirements of a good anti-coagulant dye from these results, points of importance appear to be:

(*a*) The general constitution, namely disazo direct dyes prepared from tetrazotized diamines coupled with aminonaphthol sulphonic acids.

(*b*) The dye must be freely soluble in water, saline and plasma.

(*c*) The dye must not diffuse out of the plasma too freely.

(*d*) There is no relationship between the anti-coagulant activity and the molecular size.

(*e*) Alterations in the chemical structure, as in the case of chlorazol fast pink BKS and S.D.2, suggest that chemical configuration plays a definite part. Whether this results in alteration in the chemical affinity of the dye for the components of the blood-clotting system, or whether alteration in physical properties consequent upon this change of structure impairs the physicochemical balance of the colloids of plasma, is impossible to say on the evidence at our disposal.

Summary.

1. Some azo direct dyes have been examined for their effect on the coagulation time of blood.

2. Inhibition of blood clotting is a property shared by a number of these dyes.

3. The best are all disazo direct dyes prepared from tetrazotized diamines coupled with aminonaphthol sulphonic acids.

4. The most efficient anti-coagulants obtained were chlorazol fast pink BKS, its isomer S.D.2 and an exceptional specimen of Colour Index No. 518, marketed as chicago blue 6B.

5. Chemical impurities in the dye influence the action very markedly, so that purification is essential in any dye which is being used for this purpose.

We are indebted to Prof. H. P. Gilding for some of his original sample of chicago blue 6B and to Dr A. S. Parkes, for placing at our disposal certain figures he obtained in regard to the action of chlorazol sky blue FF and chicago blue 6B. Further, we desire to thank Dr F. L. Pyman, of Messrs Boots Pure Drug Co., for purifying and analysing the samples of dyes used it this work, and Messrs Imperial Chemical Industries, Ltd. (Dyestuffs Group), for supplies of dyes of normal dyeing standard and for making specially the two dyes S.D.1 and S.D.2, which are not usual commercial products. Without the generous assistance of these firms the progress of this work would have been greatly delayed.

We wish also to record our indebtedness to Prof. B. A. McSwiney, for many helpful suggestions.

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THE EFFECT OF PARATHYROID HORMONE AND OF IRRADIATED ERGOSTEROL UPON THE CALCIUM CONTENT OF THE PAROTID SALIVA OF THE DOG.

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Baxter [1932, 1933] showed that the inorganic composition of the parotid saliva of the dog varied with the stimulus used, and that the saliva secreted by means of pilocarpine stimulation had a lower concentration of calcium than the secretion stimulated by means of meat powder. He considered pilocarpine as a special artificial stimulus which provoked a particular activity of the salivary glands different from that activated by normal reflex stimulation.

De Beer and Wilson [1932] have shown recently in acute experiments, using pilocarpine as a stimulus for the secretion, that the intravenous injection of calcium chloride caused an increased concentration of calcium in the parotid saliva of the dog, while the injection of sodium chloride, potassium chloride or potassium carbonate had little effect in altering the composition of the saliva. Werther [1886] found the concentration of calcium in the parotid saliva of the dog to be approximately twice that of the serum calcium.

Hubbel and Bunting [1932] found no consistent changes in the calcium or phosphorus concentration of human saliva following viosterol treatment, and Bunting and Wixon [1917] were unable to alter the calcium content of human saliva by feeding a high calcium diet for a period of 2 weeks. Their values for the calcium content of human mixed saliva were approximately the same as for the serum calcium. Clark and Shell [1927] also came to the conclusion that there was no correlation between the diet and the composition of human saliva.

In the course of some work we have been doing in this laboratory on the effect of alterations in the serum calcium level upon the conditioned reflexes of dogs with permanent parotid gland fistulæ, we have had the

opportunity to study the effect of these two potent serum calcium raising principles (namely, parathyroid hormone and irradiated ergosterol) upon the calcium content of the parotid saliva.

The authors are indebted to the Otosclerosis Committee of the American Otological Society for financial assistance; to Eli Lilly and Co. for the parathormone; and to Mead Johnson and Co. for the irradiated ergosterol used in these experiments.

Methods.

The saliva was collected and the solutions administered by methods devised by Pavlov and adequately described by Baxter [1932]. The various stimuli were given to the dog continuously for a definite length of time—usually 3 or 10 min., depending upon the volume secreted—and then an interval of 10 min. was allowed before the stimulus was repeated. In the case in which pilocarpine hydrochloride was used, the drug was injected intravenously (0·3 mg./kg. body weight) and the collecting tube was attached immediately. In these experiments it was found that the rate of the secretion fell off rapidly after about 4 min., hence we took as our first sample the quantity secreted in this time and as the second the amount which was secreted in the following 10 min. At the end of this time the secretion had practically ceased. The calcium was determined in the saliva by the method of Krasnov, Karshan and Krejci [1932], and the method of Clark and Collip [1925] was used for the serum calcium.

The parathyroid hormone was injected intramuscularly and the saliva collected 14–16 hours after the injection, or—as in the case of dog 2—when the serum calcium was increased as a result of a series of injections. The ergosterol (Mead Johnson's 10,000 X) was given in the food in doses of 0·5 c.c. daily until the serum showed a definite increase in calcium concentration. This usually required a period of 10–12 days. At no time was sufficient of either preparation given to cause overdosage symptoms.

Results.

The results are shown in Table I. The control values of dogs 1 and 2 represent the average values of four different experiments, while those under ergosterol and parathyroid hormone are single experiments. Dog 3, with a double parotid fistula, was kindly loaned to us by Dr H. Baxter, and was used as a check against the other two, since this dog had never received either parathyroid hormone or irradiated ergosterol.

TABLE I. The effect of parathyroid hormone and of irradiated ergosterol upon the calcium content of the parotid saliva.

	Volume c.c./min.		Saliva Ca mg./100 c.c.		Serum Ca mg./ 100 c.c.	Increase in saliva Ca		Increase in serum Ca
	A	B	A	B		A	B	
Dog 1								
			Meat powder.					
Control	0·93	0·94	36·7	36·5	11·5	—	—	—
Parathormone (100 units)	0·90	0·95	43·0	42·0	15·5	6·3	5·5	4·0
Irradiated ergosterol	0·70	0·65	53·2	51·0	17·7	16·5	14·5	6·2
			Sodium chloride—5 p.c. solution.					
Control	0·77	0·81	37·5	39·9	11·5	—	—	—
Parathormone (100 units)	0·90	0·95	49·8	54·0	14·9	12·3	15·1	3·4
Irradiated ergosterol	0·70	0·80	49·0	49·4	14·8	11·5	9·5	3·3
			Hydrochloric acid—0·25 p.c. solution.					
Control	0·93	0·86	41·5	39·1	11·5	—	—	—
Parathormone (100 units)	0·95	0·95	46·8	46·5	16·2	5·3	7·4	4·7
Irradiated ergosterol	0·75	0·60	43·3	40·0	14·7	1·7	0·9	3·2
			Pilocarpine hydrochloride.					
Control	1·75	0·34	31·9	25·8	11·7	—	—	—
Parathormone (100 units)	1·87	0·60	41·5	36·4	16·5	9·6	10·6	4·8
Irradiated ergosterol	2·00	0·65	39·0	34·0	14·2	7·1	8·2	2·5
Dog 2								
			Meat powder.					
Control	3·60	3·80	37·9	36·3	11·5	—	—	—
Parathormone (100 units)	2·86	3·00	41·8	43·7	13·8	3·9	7·4	2·3
Irradiated ergosterol	4·00	4·00	41·0	42·0	15·3	3·1	5·7	3·8
			Pilocarpine hydrochloride.					
Control	3·80	0·60	30·2	27·2	11·4	—	—	—
Irradiated ergosterol	3·20	0·60	34·8	31·2	14·7	4·6	4·0	3·3
Dog 3								
			Meat powder.					
Control (right)	3·00	3·16	34·6	36·4	11·2			
Control (left)	3·16	3·16	34·4	35·2	—			
			Sodium chloride—5 p.c. solution.					
Control (right)	3·00	3·00	36·0	36·0	11·0			
			Hydrochloric acid—0·25 p.c. solution.					
Control (right)	2·83	2·66	34·2	35·2	11·0			
Control (left)	3·00	2·83	35·2	36·0	—			
			Pilocarpine hydrochloride.					
Control (right)	2·83	0·90	30·8	25·2	11·2			
Control (left)	2·66	0·95	34·0	27·2	—			

A indicates stimulus given first time. B indicates same stimulus repeated.

It is noted that the volume of saliva secreted in a minute for each stimulus is quite constant for each dog, for the first and second trials respectively, except in the case of pilocarpine where the rate of secretion falls off rapidly during the last 10 min. of the experiment. The control values for the concentration of calcium in the saliva vary with the stimulus used, and in all cases are found to be over three times the serum

calcium, except in the case of pilocarpine where a lower concentration is found than with the other stimuli. When the serum calcium is increased as a result of parathyroid hormone or ergosterol administration, the saliva calcium shows a marked increase with each stimulus used. For example, in the case of dog 1, using sodium chloride as a stimulus, the increase above the control saliva calcium is 12·3 and 15·1 mg./100 c.c. in the case of parathyroid hormone, and 11·5 and 9·5 mg./100 c.c. in the case of irradiated ergosterol; while the serum calcium values are only 3·4 and 3·3 mg./100 c.c. above the control levels. In nearly every case the rise in saliva calcium is well above the rise in serum calcium, and apparently no distinction can be made between the hormone and the ergosterol in bringing about this increase.

The rise in saliva calcium following parathyroid administration is interesting in that it may serve as a measure of the potency of parathyroid gland extracts, especially on small dogs or cats where the taking of blood presents certain difficulties.

We are unable at present to offer an explanation of the mechanism of this rise in saliva calcium following parathyroid hormone and ergosterol administration. It is interesting to note in this connection that Parhon and Cahane [1932] found the calcium content of gastric juice of dogs slightly increased as a result of parathyroid administration; also Austin and Matthews [1927] found a similar effect and prefer to explain their results on a dehydration basis rather than as a direct action of the hormone on the tissue cells. Merrit and Bauer [1930] found no consistent changes in the calcium content of the cerebro-spinal fluid or aqueous humour following increased serum calcium as a result of parathyroid administration. Pugsley and Selye [1933] have shown that the increased serum calcium and increased calcium excretion in the rat following parathyroid administration can be accounted for by a direct action on the bone cells.

Summary.

1. The saliva secreted by the parotid gland of the dog by means of meat powder, sodium chloride or hydrochloric acid was found to have a calcium concentration of over three times the serum calcium, while the saliva secreted by means of pilocarpine hydrochloride (0·3 mg./kg.) stimulation had a lower concentration of calcium than that stimulated by the other methods.

2. An increased serum calcium brought about by parathyroid hormone or irradiated ergosterol was accompanied by an increase in the saliva calcium which exceeded the rise in the serum calcium.

3. The rise in saliva calcium following parathyroid hormone administration offers a means of measuring the potency of parathyroid gland extracts, where the taking of blood presents certain difficulties.

The authors are deeply indebted to Prof. B. P. Babkin and Prof. J. B. Collip for much helpful criticism and advice throughout the course of this work.

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NERVES AND NERVE ENDINGS IN THE VISCERAL PLEURA OF THE CAT.

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(*Received September* 5, 1933.)

The presence of nerves in the visceral pleura was mentioned by Remak, Bourgery and by Kolliker about the middle of the last century, but few thorough anatomical studies had been carried out until Larsell began his work on the innervation of the lungs and pleura. Hoffman [1920], in studies on the human pleural membrane, pointed out the marked contrast between the richly innervated parietal pleura and the sparsely innervated visceral pleura but made no reference to nerve endings. Larsell [1921, 1922, 1923, 1928] and his colleagues in the rabbit, dog and pig were able to demonstrate nerves and nerve endings in the visceral pleura by means of an *intra vitam* methylene-blue technique. In these lower animals they found that the nerves from the pulmonary plexuses enter the lung to form two main plexuses, the bronchial and the peri-arterial. From the latter, myelinated nerves pass to the visceral pleura. These fibres run for some distance in the pleural membranes as fairly compact trunks and then break up into smaller branches or into individual fibres. The main trunks follow the margins of the interlobar fissures and the smaller branches do not wander far from this general region. They found that the endings of these nerves are small and simple in the rabbit but larger and more elaborate in the dog. They are seen at the ends of the myelinated fibres in the form of branching twigs which end in knob-like expansions. The myelin sheath extends to within a short distance of the nerve ending. Such endings are found near the margins of the lobes especially near the hilum, and they become fewer and farther apart in proportion to the distance from the hilum. None were found on the costal or diaphragmatic surfaces of the pulmonary pleura. The nerve fibres probably come from the cells of the upper thoracic root ganglia through the white rami communicantes of the corresponding thoracic nerves, the sympathetic trunk, the middle and

inferior cervical ganglia and finally through the pulmonary plexuses. Larsell holds the opinion that the vagus does not have any part in the innervation of the visceral pleura, because with Mason [Larsell and Mason, 1921] he showed that in the rabbit after section and degeneration of the vagus on one side, there remain as many nerve endings (of the type he describes) in the visceral pleura on the affected side as were found on the sound side.

In a more recent communication with Dow [Larsell and Dow, 1933] on the innervation of the human lungs and pleura, Larsell showed that the plexuses and ganglia of the human lung are in general similar to those of the dog and rabbit, but in addition nerve trunks are present in the interlobular septa. Fibres from these nerve trunks reach the visceral pleura especially near the hilum, but no nerve terminations of the afferent type are found. He considers this failure to demonstrate nerve endings to be due to difficulty of staining rather than to their absence.

In my studies of the innervation of the visceral pleura of the cat I used a modification of Larsell's *intra vitam* methylene-blue technique, and I have been able to demonstrate in the pleura nerves and nerve endings which differ markedly in type and position from those which Larsell has described in the dog and rabbit. The nerve endings are of a type which has not been previously described although they bear some resemblance to the smaller encapsulated endings found in the mesentery. They are found in the pleura all round the lobe from the hilum to the apex and are quite as numerous at the apex as they are at the hilum. They are seen in large numbers on the costal and interlobar surfaces of the lobe as well as near the hilum.

The nerve fibres in the pulmonary pleura of the cat are also more numerous than those which Larsell describes in the dog and rabbit. In the cat three different categories of nerve fibres are seen. Again, they are found all round the lobe and not concentrated near the hilum. (*a*) A fine plexus of unmedullated nerves (Pl. I, fig. 2 and Pl. II, *A*) is seen just under the mesothelial surface of the pleura and running parallel with it. The endings about to be described in detail are attached to the nerve fibres of this plexus. (*b*) Larger bundles of myelinated nerves (Pl. II, *B*) which appear to be coming from the interlobular septa; when they reach the pleura they run in a diagonal direction across it, but at first more or less parallel with it, finally ending near the mesothelial surface. From the main trunk smaller branches are given off at intervals to form a fairly fine network. So far I have not been able to trace any connection between these bundles of myelinated nerves and the fine superficial

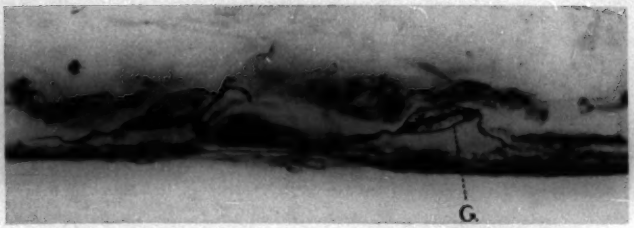


Fig. 1. Photograph of two bulbs with nerve fibres near the epithelial surface of the viscera. pleura. Cat. *Intra vitam* methylene blue. ×1312.

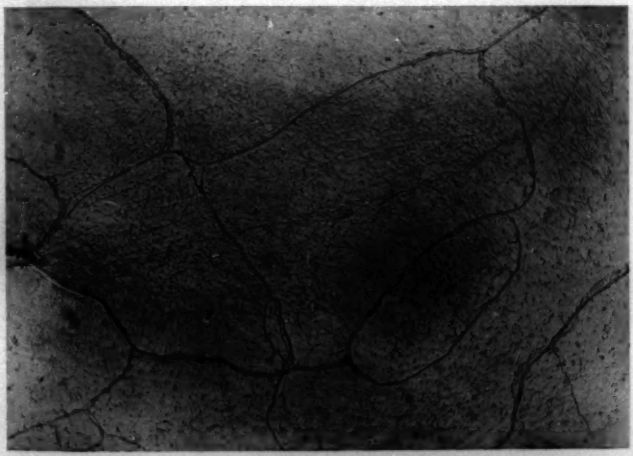
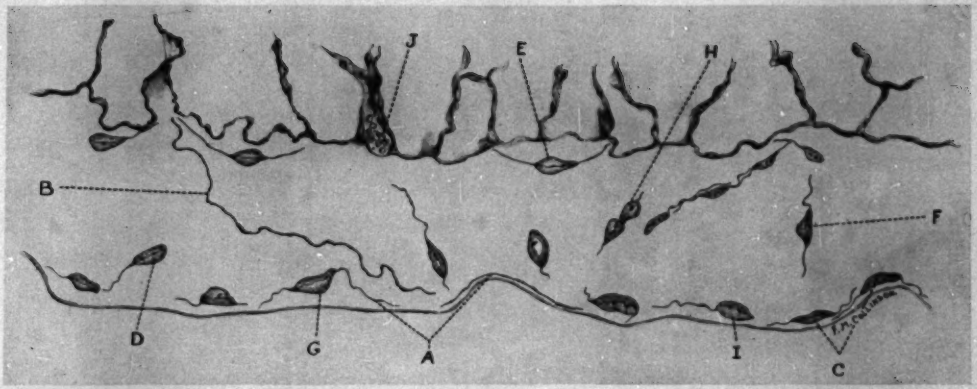


Fig. 2. Photograph of fine nerve plexus near the mesothelial surface of the visceral pleura of the cat. *Intra vitam* methylene blue. Pleura stripped off the lung and mounted flat. ×64.

To face p. 102

To face p. 103



Composite drawing of several microscopic fields of the visceral pleura of the cat showing the various positions in which the end corpuscles and nerves are found. (For explanations of letters *A–J* see text, pp. 2 and 3.) *Intra vitam* methylene blue preparation. Paraffin sections 20–60. × about 800.

plexus just mentioned. (c) In the third category are the nerves accompanying the small blood vessels found in the pleura, one group running close to and parallel with the vessel and the second group consisting of fine varicose fibrils on the actual vessel wall. Nerve trunks (Pl. II, *J* in cross-section) are present in the interlobular septa.

The nerve endings (see Pl. I, fig. 1 and Pl. II) are leaf-like corpuscles about 15 μ long and 7 μ wide, most of which are seen lying immediately under the mesothelial surface of the pleura, their long axes being parallel with the surface (Pl. II, *C*). Others, attached to fibres coming from the fine superficial nerve plexus, are seen placed at about an angle of 45° to the surface and deeper in the substance of the pleura (Pl. II, *D*). In the latter instance the corpuscle is wider and not so flattened as those lying just beneath the surface. A few similar corpuscles attached to nerve fibres are found near the pulmonary edge of the pleura (Pl. II, *E*), and occasionally a corpuscle is seen on a fibre running across the pleura with its long axis at right angles to the surface (Pl. II, *F*). Those near the surface are found in groups of three or four all round the periphery of the lobe.

The relationship of the fibres to the corpuscles varies. In some instances the fibre is seen to run parallel with the mesothelial surface, and then swing away into the substance of the pleura, runs through a corpuscle and returns to the surface to run parallel with it (Pl. II, *G*). In other instances the fibre runs through a corpuscle into another placed close to it (Pl. II, *H*), somewhat like the arrangement described by Sheehan [1933*a*] in the Pacinian corpuscles of the mesentery. Finally in many others the fibre appears to end definitely in the corpuscle (Pl. II, *I*).

Each corpuscle is ovoid in shape with pointed ends. The nerve fibre on entering the corpuscle appears to run into a spindle-shaped area and divides into a number of finer fibrils. I have not yet been able to determine whether this central spindle is made up entirely of nerve fibrils or whether it is a capsule enclosing the actual nerve ending. One definite central fibril runs through the centre of the spindle, and on its course is frequently seen a larger deeply staining varicosity placed usually at the junction of the distal one-third with the proximal two-thirds of the length of the corpuscle. Outside the central spindle the corpuscle is packed with large non-staining granules. The outer envelope is thin and sharply defined.

The origin of these nerve endings has still to be determined. At first sight they appear to be afferent sensory endings and they are probably concerned in the reception of afferent impulses for the reflexes of respiration. In structure these nerve endings show a certain resemblance to the

group of encapsulated nerve endings which includes the Vater-Pacinian corpuscles, Golgi-Mazzoni bodies and the corpuscles of Herbst. In the mesentery of the cat the bulbs are of two types [see Sheehan, 1933*b*], the first being the large laminated Pacinian corpuscles which are about ½–1 mm. in length and can be seen with the naked eye, and the second type being smaller (about 1/100 mm. long) non-laminated bulbs. The end-bulbs in the visceral pleura just described are about 15 μ in length and can only be seen satisfactorily with an oil immersion lens. It is clear that if these corpuscles in the visceral pleura are to be placed in the same category of encapsulated nerve endings there is a marked difference in the relative sizes of the mesenteric and pleural end-bulbs in the cat. Those in the pleura are all approximately of the same size. A further communication as to the origin and probable function of these pleural end-bulbs will be made.

Summary.

Nerves and nerve endings in the visceral pleura of the cat are described. Their distribution is more extensive than that previously described in man and the lower animals. The encapsulated endings, which show points of resemblance to those found in the mesentery of the cat, differ from them in that they are smaller and of comparatively constant size. Such encapsulated endings have not been previously described in the visceral pleura.

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THE EFFECT ON THE REPRODUCTIVE ORGANS OF THE RAT OF PROLONGED TREATMENT WITH OVARY-STIMULATING SUBSTANCES.

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(*Received September* 5, 1933.)

I. Introduction.

Evans [1924] suppressed the œstrous cycle of rats by the injection of large amounts of saline extract of ox anterior pituitary glands, which caused wholesale luteinization of the Graafian follicles. This result has been confirmed by many workers [see Katzman, 1932]. Another type of response to anterior pituitary administration was subsequently demonstrated by Smith and Engle [1927] and Zondek and Aschheim [1927], who found that intramuscular inplantation of fresh hypophyseal tissue caused premature œstrus and ovulation in the immature animal and "superovulation" in the adult. Thus both the major phases of the ovarian cycle, *i.e.* ovulation and luteinization of the follicular epithelium, can be reproduced by administration of anterior pituitary substance. Most crude extracts of anterior pituitary lobe, and also of urine of pregnancy in which similar substances were later discovered [Zondek and Aschheim, 1928], have since been found to give both the luteinizing and ovulation-producing effects in varying proportions. It is evident therefore that striking changes are likely to be produced in the ovary by the prolonged administration of such extracts. Firstly, the ovary might be completely disorganized by masses of normal and atretic corpora lutea of all ages and, secondly, the supply of young follicles might become exhausted. Various authors have reported the actual effects produced by such treatment [Katzman, 1932].

Fluhman [1932] noticed a marked increase in the ovarian weights of immature rats as a result of prolonged injection of an ovary-stimulating substance obtained from the blood of pregnant women. On the other hand, he did not obtain the same result with a similar extract made from sheep pituitary glands [1933], but found a rapid increase in ovarian

weights for the first 5 days, after which there was no significant increase in spite of continued administration up to 20 days. This he points out agrees with the view of Evans and Simpson [1929] that the hormone of the anterior pituitary gland is not identical with that from urine of pregnancy. Katzman, working with crystalline theelin and theelol and a non-crystalline luteinizing extract, states that these substances do not permanently affect the normal ovarian function. The luteinizing substance, however, inhibited œstrus.

The author, at McGill University, injected a series of immature rats with Collip's A.P.L. fraction from human placenta, for varying lengths of time from 10 to 60 days, starting at approximately 25 days of age. Small groups of these animals were killed at intervals of approximately 10 days and the ovaries weighed. There was a progressive increase in ovarian weight with time of injection until a certain point was reached, after which there was a rapid decline. It was thought that possibly the ovary had been over-stimulated and had become exhausted in a comparatively young animal. This experiment was repeated on another series of rats injected with untreated pregnancy urine and a similar type of curve was obtained, although the increase in ovarian weight was not so marked as in the case of the animals treated with A.P.L.

In the experiments described in the present paper a study was made of the effect of prolonged injection of extracts of anterior pituitary gland and of pregnancy urine on the reproductive organs of the adult female rat.

II. Methods and Materials.

Animals. Fifty adult female rats, divided into groups of 24 and 26 animals, were fed on an adequate standard diet and housed two animals in each cage. Later a male was placed in each cage. Vaginal smears were made daily for 2 weeks before treatment was begun to obtain information of the normal œstrous cycle of each animal. After this preliminary control period, the rats were injected daily for a period of about 6 weeks (see Figs. 1 and 2). The male rats were added to the cages after the 18th day of injection. Meanwhile smears were taken from all the females until killed. The ovaries and uteri of the treated animals were fixed, weighed and studied histologically.

Extracts. The stock supply of the material from pregnancy urine was made according to the technique of Hill and Parkes [1930] and kept in the form of a dry powder in the cold room. Small amounts of this powder were put into solution when required—usually a solution was made up to

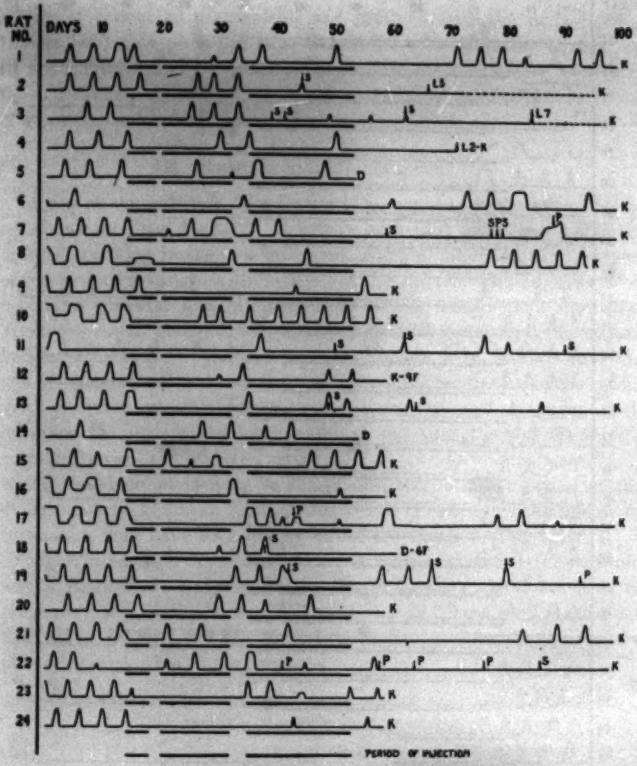
RAT NO.
DAYS 10 20 30 40 50 60 70 80 90 100
PERIOD OF INJECTION
[♂'s added to all cages on 32nd day]
K, killed, S, sperm;
L, litter; D, died;
P, vaginal plug.

Fig. 1. Vaginal reaction, matings and pregnancies in rats treated with anterior pituitary extract.

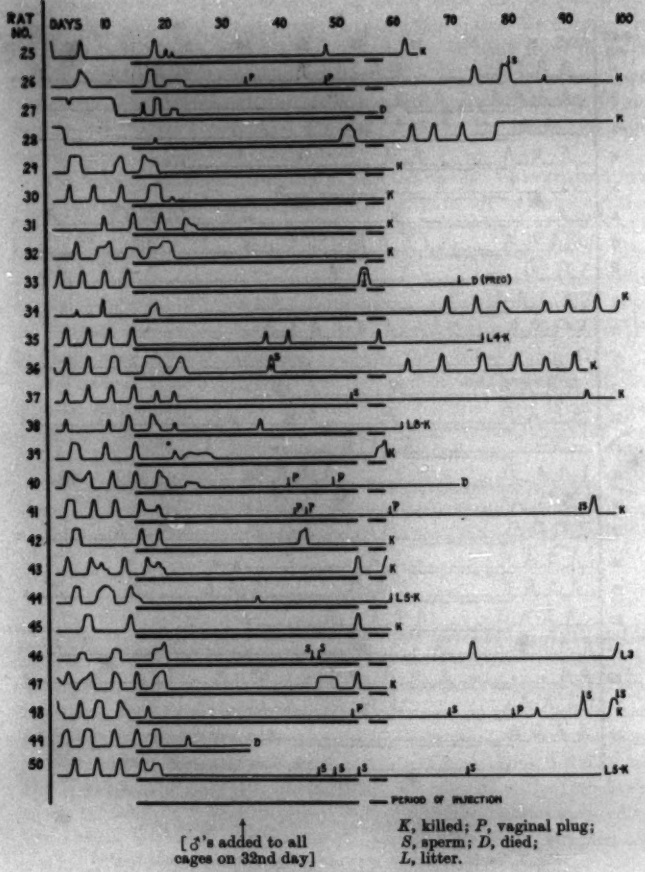

Fig. 2. Vaginal reaction, matings and pregnancies in rats treated with extract of pregnancy urine.

last about 5 days. The extract of anterior pituitary material first used was made according to the procedure of Hill and Parkes [1931]; later a supply of desiccated ox material was obtained from the British Drug Houses, and this procedure was changed for the pyridine method of Fevold, Hisaw and Leonard [1931], which was used during the rest of the experiment. A stock supply of this material was also kept in the form of a powder and made up into solution as required.

III. Results and conclusions.

Effect on vaginal cycle. The results of the vaginal smear examination are shown in Figs. 1 and 2. It will be seen that there was a slightly different reaction in the two groups of animals. Injection of extract of pregnancy urine caused a stimulation of the œstrous reaction, the cycles becoming irregular and in many cases lasting longer than normally. When this initial period of stimulation passed off (5–10 days), the smears became dioestrous in type and remained thus in most animals until the end of injection, after which there was a partial recurrence of the cycle. With pituitary extract a different reaction was obtained; coincident with injection, the cycle was quickly inhibited, but after a period of approximately 10 days there was a return of the cycle in many animals (see Fig. 1) which lasted about 10 days or longer, and then another period of the dioestrous type of smear. The sudden return of œstrus, in the middle of treatment, may have been due to one of the extracts being inactive.

Effect on fertility. As a result of treatment in both groups of rats there was a marked decrease in fertility as judged by the occurrence of pregnancy and litters born (see Figs. 1 and 2). These results are summarized in Tables I and II. Matings were diagnosed by the finding of a plug or spermatozoa in the vagina.

Table I. Showing the number of pregnancies and litters born in animals treated with anterior pituitary extract.

No. of animal	No. in litter	Conceptions		Matings detected	
		During injection	After injection	During injection	After injection
2	5	+	.	.	.
3	7	.	+	+	+
4	2	.	+	.	.
7	.	.	.	.	+
11	.	.	.	+	+
12	9 embryos	+	.	.	.
13	.	.	.	+	+
18	6 embryos	.	+	+	.
19	.	.	.	+	+
22	.	.	.	+	+

From Table I it will be seen that of the 24 animals treated with pituitary 10 mated (about 42 p.c.); of these five became pregnant (21 p.c.), but only two during the time of treatment (8 p.c.).

TABLE II. Showing the number of pregnancies and litters born in animals treated with extract of pregnancy urine.

No. of animal	No. in litter	Conceptions		Matings detected	
		During injection	After injection	During injection	After injection
26	.	.	.	+	+
33	Pregnant at autopsy	.	+	.	.
35	4	+	.	.	.
36	.	.	.	+	.
38	8	+	.	.	.
40	.	.	.	+	.
41	.	.	.	+	+
44	5	+	.	.	.
46	3	.	+	+	.
48	.	.	.	+	+
50	5	.	+	+	+

From Table II it will be seen that of the 26 animals treated with extract of pregnancy urine 11 mated (42 p.c.); of these six became pregnant (23 p.c.), but only three during the time of treatment (11·5 p.c.). Taking Tables I and II together we find: of 50 animals 21 mated (42 p.c.) and 11 became pregnant (22 p.c.), of which five conceived during the time of injection (10 p.c.). Even allowing for the usual degree of infertility, the percentage of pregnancies is very low.

Effect on ovary and uterus. The effect of the extracts on the weight of the ovaries and uterus is shown in Table III. It will be seen from an examination of these figures that the extract of pregnancy urine had much more effect on the weight of uterus and ovaries than had the pituitary material. Moreover, amongst the animals treated with pituitary, the size of the ovaries and uteri is little different in those killed at the end of injection compared with those killed later. A different state of affairs is seen after the injections of pregnancy urine. Ovaries weighing more than 100 mg. are found in only two non-pregnant animals killed at an interval after the last injection. On the other hand five of the 11 killed immediately after the last injection had very large ovaries. In view of the previous results obtained at McGill, it seems probable that the maximum size of the ovaries had been passed by the end of injection. One animal dying after 20 days' treatment actually had ovaries weighing 278 mg.

TABLE III. Showing body, uterus, and ovary weight of animals treated with anterior pituitary and pregnancy urine extracts.

Animals treated with Anterior Pituitary					Animals treated with Pregnancy Urine				
No. of animal	Body wt. g.	Uterus wt. g.	Ovary wt. g.	Killed no. of days after last injection	No. of animal	Body wt. g.	Uterus wt. g.	Ovary wt. g.	Killed no. of days after last injection
1	210	0·317	0·077	46	25	140	0·118	0·051	6
2	210	0·266	0·055	42	26	175	0·470	0·127	40
3	160	0·127	0·035	44	27	170	1·220	0·227	0
4	175	0·483	0·075	18	28	180	0·600	0·255	40
5	—	0·220	0·040	0	29	—	0·425	0·135	2
6	175	0·433	0·032	46	30	135	0·415	0·285	0
7	150	0·129	0·032	46	31	185	0·426	0·146	0
8	195	0·571	0·084	41	32	195	0·477	0·221	0
9	180	0·189	0·041	6	33	145	*3·955	0·085	died
10	160	0·807	0·053	6	34	200	1·410	0·097	41
11	—	0·503	0·060	46	35	205	1·550	0·084	17
12	190	0·679	0·046	6	36	180	0·250	0·043	35
13	170	0·420	0·067	46	37	230	0·511	0·114	40
14	125	0·133	0·019	died	38	200	1·990	0·047	3
15	170	0·298	0·034	6	39	130	0·380	0·046	0
16	175	1·102	0·030	6	40	125	—	—	died
17	180	0·503	0·070	46	41	180	†6·200	†0·280	40
18	200	—	—	died	42	140	0·125	0·032	0
19	180	0·555	0·050	46	43	160	1·004	0·054	0
20	160	0·595	0·037	46	44	—	0·694	0·070	1
21	165	0·197	0·026	46	45	160	0·215	0·032	1
22	170	0·364	0·054	45	46	160	1·820	0·076	40
23	140	0·210	0·028	6	47	—	0·251	0·064	0
24	165	0·250	0·050	6	48	175	0·430	0·075	40
					49	—	0·363	0·278	died
					50	200	0·325	0·132	37
Average wt.	173	0·407	0·047	—	—	171	0·673	0·116	—
Wt. per 100 gm. Body wt.		0·235	0·028	—	—	—	0·394	0·068	—

* Pregnant; weight not included in averages.
† Uterus with tumour; ovary, cystic; weights not included in averages.

Histology of the ovaries. Histologically, the ovaries of the animals treated with anterior lobe extract are not markedly abnormal, even when autopsy was performed immediately after the last injection.

The ovaries of the animals treated with extract of pregnancy urine and killed at this time, however, show the usual multiplicity of atretic and normal corpora lutea, and normal follicles of appreciable size are rare. These ovaries, too, appear to have passed the peak of activity, as very large numbers of old regressed corpora lutea are present. The ovaries

of the animals treated with pregnancy urine killed at an interval after injection are much more normal in appearance, the number of corpora lutea being reduced and normal follicles abundant.

IV. Summary.

1. Adult female rats were injected for a period of about 6 weeks with anterior pituitary extract (24 rats) and with extract of pregnancy urine (26 rats).

2. During the period of injection there was partial or entire inhibition of the vaginal cycle, and a marked decrease in fertility.

3. Most of the animals left for an interval after injection showed recurrence of the vaginal cycle.

4. The histological findings suggest that the peak of reaction to the treatment occurred before the end of injection.

5. The ovary is probably able to recover comparatively soon from the effects of prolonged treatment with stimulating substances.

The writer wishes to express his thanks for the assistance of Dr A. S. Parkes in histological studies and the preparation of the manuscript.

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CARBONIC ANHYDRASE. ITS PREPARATION AND PROPERTIES.

By the late N. U. MELDRUM and F. J. W. ROUGHTON.

(*From the Physiological and Biochemical Laboratories, Cambridge.*)

(*Received August* 2, 1933.)

Introduction.

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Volume 80, page 34

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From 1917 to 1921 the problem was worked at intensively by numerous British physiologists, with the result that at the end of this period the bicarbonate theory, in the view of most writers, had established a decided lead over its rival. Thereupon the scene shifted to America, and there followed an exhaustive study of the acid-base equilibria in blood by van Slyke and various colleagues. These workers gave full data as to the titration curves of oxyhæmoglobin and reduced hæmoglobin, which, together with their data upon the CO_2 dissociation curves, seemed to show that under physiological conditions the amount of CO_2

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Introduction.

There have been two main theories as to the mode of carbon dioxide transport in blood: (*a*) that carbon dioxide is carried from the organs of the body to the lung mainly in the form of bicarbonate, and that when the blood reaches the lung the proteins of the blood acting as weak acids (expressible by the formula HP) convert the bicarbonate into carbonic acid (H_2CO_3) which in turn dehydrates to water and dissolved carbon dioxide. The latter then volatilizes as CO_2 gas into the air spaces in the lung from which it is eliminated by expiration; (*b*) that in addition part, and possibly the more important part physiologically, of the carbon dioxide is carried in direct reversible combination with the blood proteins. Of these theories (*a*) has been called the bicarbonate theory and has had the support of Zuntz (quoted by Haldane, 1922), Parsons [1919, 1920], Poulton [1920], L. J. Henderson [1928], Peters and van Slyke [1931], Stadie [1928, 1931] and their co-workers. The direct combination theory (*b*) has, on the other hand, been sponsored by Bohr [1909], Buckmaster [1917 *a*, *b*], Bayliss [1924], Mellanby and Thomas [1920], and by later workers to whom reference will be made below.

From 1917 to 1921 the problem was worked at intensively by numerous British physiologists, with the result that at the end of this period the bicarbonate theory, in the view of most writers, had established a decided lead over its rival. Thereupon the scene shifted to America, and there followed an exhaustive study of the acid-base equilibria in blood by van Slyke and various colleagues. These workers gave full data as to the titration curves of oxyhæmoglobin and reduced hæmoglobin, which, together with their data upon the CO_2 dissociation curves, seemed to show that under physiological conditions the amount of CO_2

in blood in forms other than CO_2, H_2CO_3 or HCO_3^- is negligible. It will, however, be noted below that both in this work, and in that of Stadie and his collaborators [1928, 1931], there are certain features which can only be explained by the simple bicarbonate theory, with the aid of rather forced assumptions. These apparent anomalies readily fall out if a certain proportion of carbon dioxide exists in some form other than CO_2, H_2CO_3, or HCO_3^-, *e.g.* in direct combination with the blood proteins.

Up till 1925, attention had been given solely to the equilibrium states of the process, but in that year Hartridge and Roughton, quoted by L. J. Henderson [1925], following on their measurements of the velocity of oxygen combination and dissociation from hæmoglobin, pointed out the desirability of studying the kinetics of the carbon dioxide processes in blood. In 1926 this aspect was taken up by Henriques with a startling outcome.

According to the bicarbonate theory the detailed chemical reactions which lead to CO_2 evolution in the lungs are as follows:

$$HP\,(\text{protein acid}) + NaHCO_3 \rightarrow NaP\,(\text{protein salt}) + H_2CO_3, \qquad \ldots\ldots(i)$$

followed by

$$H_2CO_3 \rightarrow CO_2 + H_2O. \qquad \ldots\ldots(ii)$$

Of these reactions (i), being purely ionic, is very rapid, but (ii) is known from the work of Thiel *et al.* [1913 *a*, *b*], Faurholt [1924], and the recent paper of Brinkman, Margaria and Roughton [1933] to be rather slow. On the bicarbonate theory reaction (ii) must therefore limit the rate of CO_2 escape in the expired air. Using the velocity constants of previous authors, Henriques (and others independently) calculated that the rate at which CO_2 could be formed by reaction (ii) under physiological conditions would be far less than that actually observed in the expired air. Hence he concluded that either one or both of the following conditions must obtain: (*a*) there must be a catalyst for the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ in the blood; (*b*) the physiological transport of carbon dioxide in the blood must take place by some mechanism other than the bicarbonate one.

The most important of Henriques' [1928] experiments were those in which he compared the rate at which CO_2 is evolved when hæmoglobin solutions, on the one hand, and serum solutions, on the other hand, were violently shaken *in vacuo*. He noted for the first time that CO_2 came off much more quickly from the hæmoglobin solutions especially in the early stages of the shaking, whereas the rate of escape from serum was of the same order as that calculated from Faurholt's velocity constants for

the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$. He concluded that his results could not be explained by a catalyst mechanism, but required instead the existence of a very rapid reversible reaction between CO_2 and hæmoglobin direct, analogous to the reaction between oxygen and hæmoglobin. To this hypothetic complex, which he suggested might be of a carbamate type, he gave the name carbhæmoglobin. Hawkins and van Slyke [1930], however, in experiments of a similar type found distinct evidence of a catalyst, and this was clinched by Brinkman and Margaria [1931], who showed that blood or hæmoglobin, even when diluted to 1 part in 20,000, accelerated markedly the evolution of CO_2 from mixtures of bicarbonate with buffers of physiological *p*H. Finally in 1932 the present writers isolated from ox blood a white substance of which 1 part in 10,000,000 is active in accelerating the reaction. This solid extract, which is quite free from hæmoglobin and apparently also from hæmatin compounds, has the typical properties of an enzyme, and since it can be shown to be distinct from the other enzymes known to be present in blood, it has been given a special name: CARBONIC ANHYDRASE. Preliminary reports on its biochemical properties have already been given by us elsewhere [Meldrum and Roughton, 1932 *a*, *b*, *c*], and a more complete account of its preparation and properties is given below.

With such large amounts of carbonic anhydrase in the red blood corpuscles it no longer seems difficult to account for the rate of CO_2 evolution in the expired air on the simple bicarbonate theory. This does not, however, prove that the bicarbonate mechanism is the sole mode of physiological transport; a definite conclusion as to the rôle of the hypothetical CO_2 protein compounds is indeed needed, not only for the sake of its own interest and for possible importance, but also to enable us to assess precisely the rôle of carbonic anhydrase in blood. For reasons to be given later, we do not regard either the recent experiments of Henriques [1928, 1931 *a*, *b*, 1933] or of Margaria [1931] as decisive or complete, and we have therefore devised and carried out a new set of experiments, which seem to us less equivocal in their indications. Our results not only point to appreciable amounts of direct CO_2 protein compounds especially at low temperatures (the amount diminishing very much as the temperature and/or the acidity is increased), but also suggest strongly that these compounds are of a carbamate type, as has indeed been hinted at by previous workers. The whole question is dealt with in a separate paper by us [Meldrum and Roughton, 1933*b*] in the present *Journal*.

Scope of the present paper.

We shall start by describing the method of measuring the catalytic activity, and pass thence to the various methods of separating the enzyme from hæmoglobin and the means adopted for its further purification. After summarizing the evidence that carbonic anhydrase is distinct, not only from hæmoglobin, but also from some other enzymes present in the blood corpuscles, we then proceed to some of the biochemical properties of the enzyme. Here we deal, in a preliminary way, with the nature of the enzyme and some of the factors which influence its stability and activity. We have made a fairly detailed study of the effect of various poisons and inhibitors, including metallic salts from most groups in the periodic table. In the last pages we give some further notes about the distribution of the enzyme, especially in fœtal blood as compared with maternal blood; we also compare the carbonic anhydrase activity of unlaked corpuscles with that of laked corpuscles from the same blood. This last matter is obviously of great physiological importance as regards the action of the enzyme in circulating blood: our preliminary experiments show that there is need for much further study on these lines.

Section I. The method of estimating the catalytic activity of the various preparations.

The technique.

A simple means of measuring the amount of enzyme present in the various media and preparations is clearly an indispensable preliminary to a study of the isolation and properties of the enzyme. The method adopted was to measure the catalytic effect upon the rate of evolution of CO_2 from sodium-bicarbonate solutions when mixed with phosphate buffer of *p*H 6·8, with the aid of the boat apparatus of Brinkman, Margaria and Roughton [1933] as used by them in their work on the kinetics of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ in the absence of catalyst.

In one compartment of the glass boat-shaped trough (Fig. 1) are placed 2 c.c. of phosphate solution made by mixing equal volumes of *M*/5 Na_2HPO_4 and *M*/5 KH_2PO_4, whilst in the other compartment are placed 2 c.c. of *M*/5 $NaHCO_3$ dissolved in *M*/50 NaOH. The boat is attached by a well-fitting stopper and length of pressure tubing to a manometer, open at the other end to the air. Most of the experiments in this paper

were thus done at atmospheric pressure, instead of at reduced pressure as in the earlier paper.

After allowing time for temperature equilibration the boat is then shaken rapidly in a constant temperature water bath (usual temperature about 15° C.) and the evolution of CO_2 followed by noting the reading of the manometer at 0, 5, 10, 15, 30, 45, 60, 90 and 120 sec. and thereafter at minute intervals if necessary. Provided that the first half of

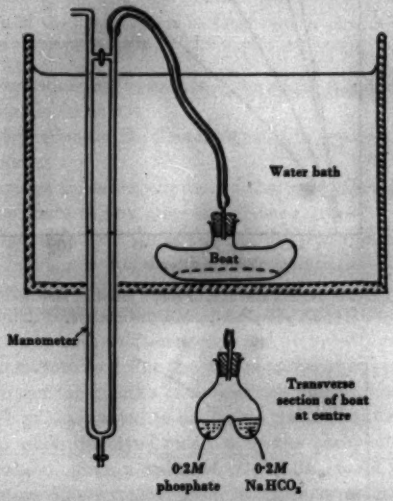

Fig. 1. Boat technique for measurement of enzyme activity.

the total CO_2 is evolved in not less than about 15 sec., diffusion from liquid to gas phase does not prove to be a limiting factor, and the rate of CO_2 evolution as observed is a true measure of the velocity of the chemical reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. A typical set of curves obtained with various amounts of goat's blood added to the phosphate-bicarbonate mixture is shown in Fig. 2 *a*.

Calculation of the activity.

In Fig. 2 *b* is plotted the average rate of CO_2 evolution during the second quarter of the process (as given by the reciprocal of the time

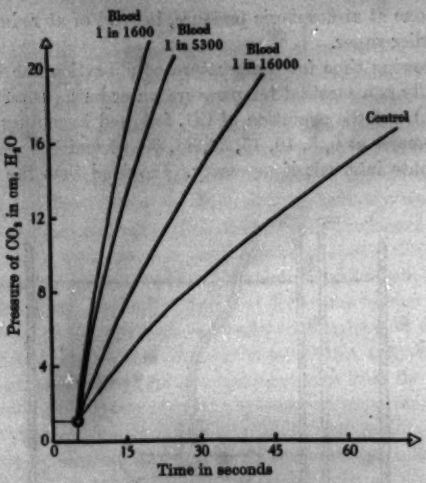

Fig. 2 a. Effect of various amounts of goat's blood on rate of CO_2 output from phosphate-bicarbonate mixture.

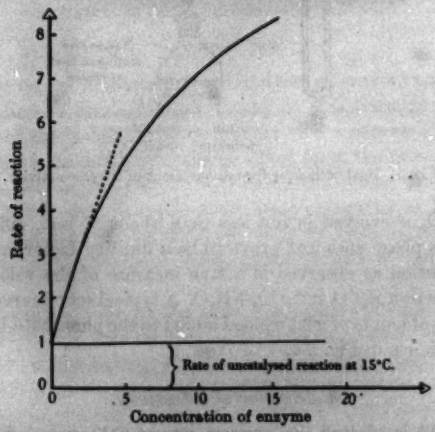

Fig. 2 b. Relation between concentration of enzyme and effect on rate of reaction. [The rate of the reaction in absence of catalyst being taken as unity.]

taken) against the concentration of catalytic material added, the samples of the latter ranging from crude blood to relatively pure material.

Let R_0 be the rate in absence of catalyst, R be the rate in presence of catalyst. Then it is clear from the curve that the fraction $(R-R_0)/R_0$ is a suitable measure of the catalytic activity, for:

(*a*) It is proportional to the amount of catalyst added, to within ± 5 p.c., provided that the rate of the process is not too fast, *i.e.* the time is not less than 10 sec.

The breakdown of the linear relation at faster rates is certainly in part due to diffusion becoming a limiting factor in addition to chemical reaction velocity.

(*b*) It is independent of the nature of the added catalyst, *e.g.* whether it contains hæmoglobin or not.

(*c*) It is independent of the dimensions of the apparatus, which need not even be known.

(*d*) It eliminates initial errors due to the time taken for the bicarbonate and phosphate to mix after the shaker starts.

(*e*) The range of the *p*H change during the period is small and constant.

In ordinary cases it makes no difference whether the catalytic material is added to the phosphate or to the bicarbonate, or if it is distributed between both solutions.

It seems premature to define the unit of carbonic anhydrase activity until a full study of the kinetics of the enzyme reactions has been made. For the present it is proposed to assign the value E to that amount of enzyme which, when dissolved in 4 c.c. of the phosphate bicarbonate mixture used above, gives a value of $(R-R_0)/R_0$ equal to 1 at 15° C. If the temperature differs slightly from 15° C., a correction can be made by applying a temperature coefficient (*i.e.* Q_{10}) of 3·6 per 10° C. for the uncatalysed reaction and a Q_{10} of 2·1 for the catalysed reaction. This temporary unit E has the convenience that ox blood (the main type of blood used in our work so far) contains on the average about $1\,E$ per c.mm.

Whenever a test was made of the catalytic activity of any solution, a control experiment with phosphate and bicarbonate solutions alone was done as well. To get the most accurate result the amount of catalytic material added should be such as to increase the rate of the process about three to four times, since the relation between enzyme concentration and rate of reaction is still linear up to this point. In some tests, unfortunately a much larger amount of catalyst was used; in view of the shape

of the curve in Fig. 2 *b* the results were correspondingly less accurate, even though the necessary corrections were applied.

The percentage yield in any given preparation from blood was calculated from a comparison of the activity of the preparation with that of the original blood diluted with an amount of water equal to the amount of aqueous fluid added to the blood in the course of the preparation.

Section II. Separation and purification of the enzyme.

Separation from hæmoglobin.

The finding of Brinkman, Margaria, Meldrum and Roughton [1932] that not all hæmoglobins are catalytically active, but that native globin prepared from an active hæmoglobin is fully active, suggested strongly that the active principle is not hæmoglobin itself, but some substance or some system which frequently, but not invariably, accompanies the hæmoglobin. That the latter is the case was settled very quickly by applying the classical methods of removing hæmoglobin with but little modification. Three methods, *A*, *B*, *C*, were tried, and all were successful. Of these the crude product of method *C* was used for nearly all the further work in this paper.

Method A. Mammalian erythrocytes (previously washed three times with isotonic saline) are lysed with water and diluted till the hæmoglobin is about 1 p.c. The aqueous solution is then saturated with chloroform and allowed to stand at room temperature in presence of excess $CHCl_3$ with intermittent shaking till all the hæmoglobin is denatured. The usual time required is about 24 hours: a temperature of 37° C. hastens denaturation but destroys the enzyme. The protein and excess chloroform are centrifuged down and the enzyme solution poured off. It usually contains of the order of 50 p.c. of the carbonic anhydrase originally present, much catalase, and traces of hæmoglobin ($<$ 1 in 5000). This method is similar to that of Tsuchihashi [1923] for preparing catalase.

Method B. By following the method of Warburg and Christian [1931] for preparing the co-enzyme for the system which oxidizes glucose-6-phosphate, carbonic anhydrase can readily be prepared. 200 c.c. washed horse erythrocytes are lysed at 0° C. with an equal volume of water and 350 c.c. of ice-cold alcohol added. Then 15 c.c. $CHCl_3$ are added to the homogeneous mixture and the whole shaken for a few minutes at 0° C. The suspension is centrifuged and the centrifugate poured into 450 c.c. alcohol and 1200 c.c. ether, when a whitish precipitate gradually falls.

The supernatant liquid is sucked off, the solid collected on a filter and washed with absolute alcohol.

This enzyme preparation is very stable, it does not readily dissolve in water, but gives a highly active and fairly clear solution when 10 mg. are ground in a mortar with 1 c.c. of $M/5$ Na_2HPO_4.

The yield is only about 7 p.c., and the activity per mg. dry weight is about 350 *E* as measured by the method of Section II, assuming that the whole of the enzyme is in solution. The latter assumption is, however, probably unfair.

Method C. To 10 c.c. washed ox corpuscles in a centrifuge tube are added 6 c.c. H_2O and 4 c.c. ethyl alcohol. The mixture is then shaken at room temperature for about a minute with 5 c.c. $CHCl_3$. On centrifuging for about 10 min. at 3500 r.p.m. a three-phasic system is formed, consisting of a top layer of enzyme solution, a central layer of denatured protein, and a bottom layer of chloroform.

The enzyme solution contains traces of hæmoglobin and much catalase. The yield is on the average about 50 p.c. of the maximum. In different cases there were considerable variations from this figure both in upward and downward directions. The cause of this is not known but may be in part explained by varying amounts of enzyme adhering to the hæmoglobin coagulum. Rubbing up the latter with water gave a solution of high activity on the one occasion when the test was made. On evaporating the enzyme solution to dryness in a vacuum desiccator a brown solid enzyme preparation is obtained containing methæmoglobin and hæmochromogen compounds. The yield is about 1 g. from 100 c.c. corpuscles.

The enzyme is extremely stable in this form and can be kept for many months even if exposed to the air. The brown solid readily dissolves in water or salt solutions to yield a clear solution, having the properties of the original (unevaporated) solution. In aqueous solution the enzyme keeps for many weeks in the ice chest. The rate of destruction varies with different samples, but in general the enzyme loses $\frac{1}{2}$ 20 p.c. activity in 5–6 weeks.

The solution of enzyme thus prepared is known in the succeeding parts of this paper as "crude chloroform" preparation, and is used as the basis for further purification.

Some two dozen preparations were made by this method, which was found to work for the blood of other mammals besides ox. In one case with horse blood an 80 p.c. yield was obtained. In the earlier cases of the series a lower proportion of alcohol was used, but this led to much

slower precipitation of the hæmoglobin, several shakings with $CHCl_3$ often being necessary before a colourless (*i.e.* hæmoglobin-free) supernatant fluid was obtained. The yield, however, was as great as when the proportion of alcohol mentioned above was used, the great advantage of the latter lying in the quickness of the method. The difference in speed of coagulation of the hæmoglobin forms, indeed, a striking demonstration: for the quickest results the proportion of 6 parts water to 4 parts alcohol should be adhered to closely.

Purification.

The further purification of the enzyme is best carried out starting from the crude chloroform preparation. Such a preparation contains two types of impurity: (*a*) dialysable; (*b*) non-dialysable.

The dialysable impurities are readily removable by ultra-filtration. For this purpose Bechhold's 6 p.c. and 4½ p.c. ultra-filtration membranes were purchased from Carl Schleicher and Schüll, and ultra-filtration by vacuum suction was found quite satisfactory, provided the fluid on the membrane is gently stirred.

The non-dialysable impurities: Attempts were first made to precipitate either the enzyme or the accompanying protein material with ammonium sulphate, but these were unsuccessful. In the case of crude hæmoglobin solutions most of the enzyme is carried down with the precipitated proteins, while in the case of the crude chloroform preparations a large and variable amount of enzyme is removed, probably by adsorption on precipitated material.

A series of adsorbents was next tried. Kaolin, permutit, four different sorts of charcoal, silk, cellulose and chalk were unsatisfactory. Very good results, however, were obtained with Willstätter's C-γ alumina cream, and with a suspension of $Ca_3(PO_4)_2$ (roughly 1 p.c. prepared by precipitation in the customary manner), filtering after each treatment with adsorbent. These adsorbents are both effective in removing selectively the other non-dialysable impurities, especially the traces of colouring (hæmatin) matter, without at the same time taking up serious amounts of carbonic anhydrase. The extent of the treatment must be adjusted for each sample.

The following is a protocol by N. U. M. of a purification by the $Ca_3(PO_4)_2$ method:

100 c.c. ox corpuscles were washed three times with isotonic saline and then treated with 80 c.c. water, 20 c.c. alcohol and 150 c.c. $CHCl_3$. The mixture was shaken for some minutes, stood overnight at 2° C.,

centrifuged, shaken and centrifuged again. The supernatant fluid was filtered, volume of filtrate $A = 108$ c.c. 25 c.c. A were treated with 20 c.c. of $Ca_3(PO_4)_2$ suspension, and the mixture filtered. Volume of filtrate $B = 40$ c.c.

30 c.c. of filtrate B were ultra-filtered on a 6 p.c. Bechhold membrane, being washed three times with about 13 c.c. water.

The residue on the membrane was made up to 40 c.c. with water = solution C.

The volume of the ultra-filtrate was 67 c.c. = solution C'.

0·05 c.c. of solution C was tested and found to have an activity = 6 E.

5 c.c. of solution C evaporated to dryness at 130° C. gave a dry weight of 1·2 mg. Therefore the activity per mg. of the solid in solution $C = 500$ E.

N. U. M.'s purest preparation was made by $Al(OH)_3$ adsorption, the steps of the process being otherwise almost the same as in the previous example. In this case details are available as to the activity per mg. dry weight at three different stages of the preparation.

Activity of original ox corpuscles per mg. dry weight = 4·6 E (assuming water content of corpuscles = 60 p.c. by weight).

Crude chloroform preparation shows 48 mg. dry weight per 5 c.c.

Activity per mg. dry weight = 90 E.

The dialysable impurities were removed by ultra-filtration and the final solution after three treatments with an equal volume of $Al(OH)_3$ cream had an activity per mg. dry weight = 1730 E. 5 c.c. of this final solution evaporated at 100–150° C. contained 1 mg. dry weight, and was thus almost the same in concentration as the final solution of the previous case, although the activity was about 3·5 times greater.

Comparison of the activity per mg. dry weight of the final preparation with that of the original corpuscles shows that a concentration of about 400-fold was attained.

The figures given show that 1 part of the pure solid (by weight) in 7,000,000 parts of solution is sufficient to double the rate of CO_2 evolution, and that during the first 15 sec. 1 g. of enzyme is responsible for the production of 825 g. of CO_2. This means a rate of 1·24 mols CO_2 per sec. per g. of enzyme.

If the enzyme is pure, and is a protein (*vide* Section III) of the minimum molecular weight found by Svedberg, viz. 34,000, then 1 mol enzyme would catalyse the formation of 4×10^4 mols CO_2/sec.

Further purification of the enzyme.

The fact that our best preparation has the same order of activity as the most active enzymes of other workers makes it perhaps unlikely that much further purification will be possible. We have not yet, however, used in a systematic way one of the classical weapons of enzyme purification, namely adsorption of the enzyme, followed by elutriation of the enzyme from the adsorbent. Preliminary work suggests that the enzyme is to some extent adsorbable by alumina C-γ cream, especially if the impurities adsorbed by the latter are first removed. Thus in two cases we were able by appropriate treatment to adsorb as much as 40 p.c. of the enzyme on alumina. We also found on one occasion that from the purest solution the enzyme was partly adsorbed by kaolin, and could be in part elutriated therefrom by means of $M/15$ Na_2HPO_4.

Fractional ultra-filtration is another possible line. Thus in one experiment the crude chloroform preparation was shaken with alumina, filtered and the filtrate then ultra-filtered through a 6 p.c. Bechhold membrane. The solution which came through the ultra-filter had an appreciable activity. In the two other cases, the crude chloroform preparation was ultra-filtered directly through a 4½ p.c. Bechhold membrane. The solution which came through had respectively 13 and 25 p.c. of the activity of the crude chloroform preparation in the two cases.

Section III. The "individuality" and some properties of the enzyme.

The evidence that carbonic anhydrase is distinct from hæmoglobin.

Earlier workers on the CO_2 catalyst in blood inclined to the view that it was identical with hæmoglobin. Against this there are now three strong arguments:

(*a*) There is no correlation between the distribution of hæmoglobin and of carbonic anhydrase in lower organisms. Examples of this lack of correlation have already been given by Brinkman, Margaria, Meldrum and Roughton [1932], and further instances occur in Brinkman's [1933] paper in the present number of this *Journal*. A striking case of the same kind will be found in a later section of this paper, where it is shown that in fœtal blood of goats the ratio carbonic anhydrase content/hæmoglobin concentration, *i.e.* E/Hb, may be only 1–2 p.c. of the value in the mother blood of the same animal.

(*b*) It is now possible by the simple methods of Section II to prepare highly active preparations of the enzyme, which are completely free from hæmoglobin.

(*c*) We have been able in the case of two species of blood of which the hæmoglobin crystallizes easily—namely, horse and rat—to effect a considerable separation of hæmoglobin from the enzyme merely by crystallization.

Thus in the case of horse hæmoglobin, a single crystallization gave a value of *E*/Hb about three times greater in the mother liquor than in the crystals. In the case of the rat sample the first mother liquor, after crystallization at 0° C., had only one-seventh the hæmoglobin concentration of the original blood, but yet had nearly the same concentration of carbonic anhydrase. Two further crystallizations gave a preparation of hæmoglobin with an *E*/Hb value only 7 p.c. of the *E*/Hb in the original blood. It seems likely therefore that by very many recrystallizations hæmoglobin might be obtained free from carbonic anhydrase as well as from catalase, as has already been done as regards the latter only.

The properties of such hæmoglobin, if not too largely contaminated with "inactive material," would be interesting to compare with those of hæmoglobin "purified" by the usual means.

Freedom from hæmatin compounds.

No trace of hæmatin compounds could be detected in the purified alumina preparations, even when examined in very thick layers by spectroscopic methods.

Distinction of carbonic anhydrase from other enzymes of the blood, etc.

(*a*) *Catalase.* There is no correlation between the distribution of catalase and carbonic anhydrase: thus, for example, there is much catalase in yeast, but usually no detectable carbonic anhydrase. Furthermore we found that our crude chloroform preparations, which contained abundant catalase as well as carbonic anhydrase, were freed entirely of catalase by means of the $Al(OH)_3$ adsorption technique. Thus the filtrate obtained after the usual alumina treatment still contained a high proportion of carbonic anhydrase, but no catalase.

(*b*) *Peroxidase.* Both the guaiacum and benzidine tests are negative, when applied to our pure preparation.

(*c*) *Oxidase.* Preliminary observations failed to detect blueing of tetramethylparaphenylenediamine, nor was any catalysis of oxidation of reduced cytochrome *c* to be observed.

So far then we feel justified in believing that carbonic anhydrase is a new enzyme, distinct from any previously described enzyme or system.

Protein tests, etc.

The simple characteristics of the solid obtained by evaporating the crude chloroform preparation to dryness have already been described. The active solid obtained by evaporating the purest solutions to dryness at low temperatures is colourless and almost transparent.

The presence of proteins in the various preparations was looked for by the usual tests. The results are shown in Table I, which also includes the α-naphthol test.

TABLE I.

A = Crude chloroform preparation.
B = Solution of solid prepared as in Section II, method *B*.
C = Purest solution ($Al(OH)_3$ filtration treatment, etc.).

Test	Result *A*	*B*	*C*
Millon's	++	++	++
Xantho-proteic	++	++	++
Sulphur	++	++	?
Glyoxylic	+	+	+
Biuret	.	.	+
α-Naphthol	+	+	+

++ = Marked reaction.
\+ = Moderate to slight reaction.
? = Doubtful.

Since in relatively pure solution the enzyme is only partially and variably precipitated by saturation with ammonium sulphate, and tends furthermore to pass through ultra-filtration membranes to an appreciable extent, it is probable that the enzyme, if a protein, is a rather small molecule.

Further work on such matters as the chemical composition of the enzyme, etc., its molecular weight, solubility and acid and base-binding properties, has been postponed until the efforts at maximum purification shall have been exhausted.

SECTION IV. THE STABILITY, SCOPE OF ACTION, AND KINETICS OF THE ENZYME.

Stability.

(*a*) *To temperature.* The stability of the enzyme, in three different grades of purity, was tested by exposing the solutions to various tempera-

tures for 30 min., and then measuring the activity as in Section I. The result was as follows:

	20° C.	55° C.	65° C.
Lysed corpuscles (1 part in 80 parts water)	No loss	30 p.c. loss	Completely destroyed
Crude $CHCl_3$ preparation	,,	—	,, ,,
Very pure $Al(OH)_3$ preparation (in water)	,,	No loss	60 p.c. loss

(*b*) *To pH.* The same three preparations were taken to various *p*H's for 30 min. periods, and then tested for activity.

	Citrate phosphate buffer			Borate buffer	*N*/100 NaOH	*N*/10 NaOH
	*p*H 3	*p*H 4	*p*H 6–10	*p*H 11	*p*H 12	*p*H 13
Lysed corpuscles	Completely destroyed	No loss	No loss	—	—	Completely destroyed
Crude $CHCl_3$ preparation	,,	,,	,,	No loss	No loss	,,
$Al(OH)_3$ preparation	,,	,,	,,	—	30 p.c. loss	,,

The scope of the reactions catalysed by carbonic anhydrase.

The reactions $CO_2+H_2O \rightarrow H_2CO_3$ *and* $CO_2+OH^- \rightarrow HCO_3^-$. These reactions, *i.e.* the reverse to that so far dealt with in this paper, are of course also catalysed by the enzyme. The boat technique was again used, but in this case a two-compartment vessel was not necessary. 4 c.c. of a suitable buffer were placed in the boat, the latter connected by one lead through its cork to the manometer, and by a second lead through a very fine capillary and a rubber tube with pinchcock to a vessel containing CO_2 gas at about 1½ atmospheres. About 25 sec. before shaking was due to begin the pinchcock was opened and CO_2 allowed to stream into the boat until a suitable pressure, as shown by the manometer reading, was reached. This usually took 15 sec.

After 10 sec. more, shaking was started and the uptake of CO_2 recorded by the manometer readings at 0, 5, 10 and 15 sec., etc.

For higher CO_2 pressures than 50 mm. Hg, the water in the manometer was replaced by mercury and the technique slightly altered.

Fig. 3*a* shows the catalytic effect of 1 part crude $CHCl_3$ enzyme in 40,000 parts solution upon the rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6 at 17° C. The reaction is accelerated about eight-fold. This may be compared with the fourteen-fold acceleration of CO_2 output produced by the same concentration of this sample of enzyme when tested by the method of Section I. At *p*H 7·6 the uptake of CO_2 occurs almost entirely through the reaction $CO_2+H_2O \rightarrow H_2CO_3$. Fig. 3*b*, however, gives the uptake of CO_2 by a solution containing 0·7 *M* $NaHCO_3$ and 0·57 *M* Na_2CO_3. The *p*H of this solution being about 10, the reaction

$CO_2 + OH^- \rightarrow HCO_3^-$ is also occurring to a marked extent according to Faurholt [1925], confirmed by Brinkman, Margaria and Roughton [1933]. One part of crude $CHCl_3$ enzyme in 40,000 parts of solution accelerates in this case, but to a less extent, viz. 1·7-fold.

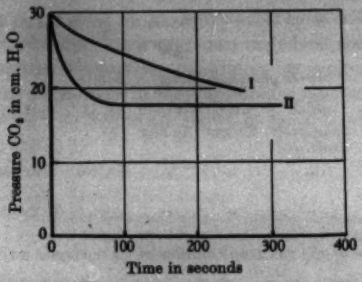

Fig. 3*a*. I = Rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6; II = Rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6, with crude $CHCl_3$ enzyme 1 in 40,000 added. Temp. 17° C.

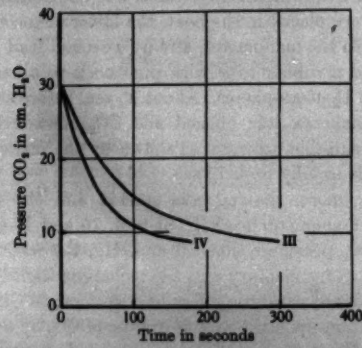

Fig. 3*b*. III = Rate of uptake of CO_2 by 0·71 *M* $NaHCO_3$—0·57 *M* Na_2CO_3; IV = Rate of uptake of CO_2 by 0·71 *M* $NaHCO_3$—0·57 *M* Na_2CO_3, with crude $CHCl_3$ enzyme 1 in 40,000 added. Temp. 17°C.

The enzyme can also be shown to catalyse CO_2 uptake by *p*H 11 solutions of Na_2CO_3, in which case the uncatalysed process occurs almost entirely *via* the reaction $CO_2 + OH^- \rightarrow HCO_3^-$.

Some more composite processes catalysed by carbonic anhydrase.

Any complex process, in which the reaction $CO_2+H_2O \rightarrow H_2CO_3$ or $CO_2+OH^- \rightarrow HCO_3^-$ is a link and is a limiting factor in the over-all speed of the whole, should be accelerated by carbonic anhydrase. Four cases of this kind were investigated, all of possible physiological bearing.

(*a*) *The solution of* $CaCO_3$ *by weak acids other than carbonic acid*, i.e. $CaCO_3+2HA \rightarrow CaA_2+H_2CO_3$, *followed by* $H_2CO_3 \rightarrow CO_2+H_2O$. If the chalk is shaken violently enough, the reaction $H_2CO_3 \rightarrow CO_2+H_2O$ tends to become a limiting factor.

A 2 p.c. suspension of $CaCO_3$ in water was shaken at 0° C. with an equal volume of acetate buffer *p*H *circa* 5, total acetate conc. 0·7 *M*. One part of crude $CHCl_3$ enzyme per 10,000 parts of suspension accelerated the solution of $CaCO_3$ three times.

(*b*) *The solution of* $CaCO_3$ *by carbonic acid to form calcium bicarbonate.* Exp.: 20 c.c. of saturated $Ca(OH)_2$ were shaken in a 70 c.c. boat with pure CO_2 at 1½ atmospheres initially.

The uptake of CO_2 was followed manometrically. There was at first a rapid uptake of CO_2 occasioned by conversion of the $Ca(OH)_2$ into $CaCO_3$ with precipitation of the latter. This was followed by a smaller slow uptake due to the partial conversion of insoluble $CaCO_3$ into slightly soluble $Ca(HCO_3)_2$, *i.e.* $CO_2+H_2O \rightarrow H_2CO_3$, followed by $H_2CO_3+CaCO_3 \rightarrow Ca(HCO_3)_2$.

That the reaction $CO_2+H_2O \rightarrow H_2CO_3$ is to some extent limiting was shown by addition of crude $CHCl_3$ enzyme 1 in 16,000.

The slow process was then accelerated about twelve times at 0° C.

The rates of solution and deposition of $CaCO_3$ are of biological interest in regard to the formation of shells and other calcareous deposits.

(*c*) *The uptake of* CO_2 *by ammonia solution.* This was also measured manometrically. There is first of all a very rapid uptake of CO_2, due to the formation of ammonium carbamate, *i.e.* $2NH_3+CO_2 \rightarrow NH_2COONH_4$. This is followed by a long slow process in which ammonium carbonate and bicarbonate are formed, the final proportions of carbamate, carbonate and bicarbonate depending upon the initial amounts of NH_3 and CO_2. Of these two processes, the rapid one, *i.e.* the carbamate formation, does not seem to be affected by carbonic anhydrase, but the slow one is much speeded up. Thus with 4 *M* ammonia, 1 part of crude $CHCl_3$ enzyme in 6000 parts accelerated the slow process ten times and even in "Ammon. Fort." (sp. gr. 0·880) the enzyme still showed appreciable activity. This is a striking instance of its robustness.

(*d*) If an equilibrium mixture of ammonium carbamate, carbonate and bicarbonate is shaken with $CaSO_4$ suspension, the carbonate and bicarbonate react very quickly with the $CaSO_4$ to form $(NH_4)_2SO_4$ and $CaCO_3$ (which is precipitated): calcium carbamate is, however, relatively soluble and does not precipitate, but with the removal of the carbonate from the sphere of action, the carbamate changes over slowly into carbonate which then precipitates. Faurholt [1925] has shown that the conversion of ammonium carbamate into bicarbonate and carbonate occurs, not by a simple addition of water as has often been thought, but by a two stage process.

$$NH_2COONH_4 \rightarrow 2NH_3 + CO_2,$$

$$CO_2 + OH^- \rightarrow HCO_3^-, \text{ followed by } HCO_3^- + OH^- \rightarrow CO_3^{--} + H_2O.$$

If so, then the precipitation of $CaCO_3$ in the slow stage should be accelerated by carbonic anhydrase. This was confirmed by estimating the amount of $CaCO_3$ precipitate formed after various periods of shaking. In the slow stage 1 part crude $CHCl_3$ enzyme in 1000 parts accelerated the process about 3·5 times at 19° C.

Examples (*c*) and (*d*) are of physiological bearing in regard to the rôle of carbonic anhydrase in the interaction of carbamates and bicarbonates. In our adjoining paper [1933 *b*] we give new evidence for the existence of protein-carbamate compounds in physiological fluids especially at low temperatures.

Some notes on the kinetics of the enzyme.

Only preliminary observations have been made so far upon the effect of varying enzyme concentration, substrate concentration, temperature, *p*H and neutral salts upon the kinetics of the reaction.

(*a*) *Enzyme concentration.* All the data so far available have been given in Section I.

(*b*) *Substrate concentration.* Experiments have been done upon the effect of varying the pressure of CO_2 upon the rate of CO_2 uptake by 0·2 *M* phosphate buffer *p*H 7·6, and by 0·7 *M* $NaHCO_3$ + 0·57 *M* Na_2CO_3 *p*H *circa* 10. In Fig. 4 the rate is plotted against CO_2 pressure both for the uncatalysed and the catalysed reaction in both cases. For the catalysed experiments 1 part crude solid enzyme per 40,000 parts solution was used at both *p*H's. From 20 mm. Hg to 380 mm. Hg the rate of the catalysed reaction is proportional to the CO_2 pressure, but falls off at higher pressure in the *p*H 7·6 case. A similar result was found for rate of uptake of CO_2 by 4 *M* NH_3 almost saturated with NaCl; in this

case, owing to the depressing action of the strong NaCl, 1 part of the same crude solid enzyme per 800 parts of solution had to be used. This indicates that the Michaelis constant of the enzyme [see Haldane, *Enzymes*, 1930] is small, both in conditions where the activity of the enzyme is high, and also where the activity is low.

The uptake of CO_2 is a far more suitable case for studying the effect of varying the concentration of substrate than is the output of CO_2, for with the boat technique the pressure of CO_2 can be varied from 10 mm. Hg to 1500 mm. (*i.e.* 150-fold), whereas the concentration of $NaHCO_3$

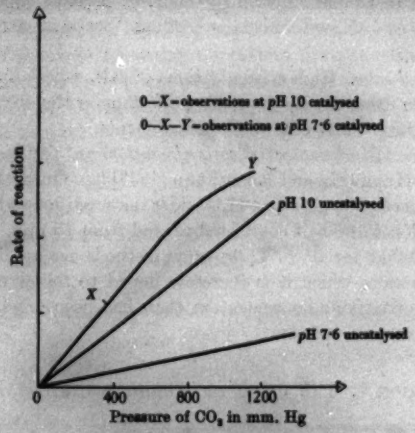

Fig. 4. Relation between substrate concentration and accelerating effect of enzyme.

cannot be conveniently varied more than about fifteen-fold. Furthermore, in the latter case with high concentrations of $NaHCO_3$ activity effects and other "neutral salt" effects, cause serious disturbances, but with high pressures of CO_2 in uptake experiments these troubles do not arise.

Only one experiment has so far been done on CO_2 output with varying substrate concentration. In this it was found that 1 part crude $CHCl_3$ enzyme in 8000 accelerated CO_2 output from 1·0*M* $NaHCO_3$ + 1·0*M* phosphate buffer *p*H 6·8 three times, whereas 1 part of the same enzyme in 40,000 accelerated CO_2 output from the usual 0·2*M* $NaHCO_3$ + 0·2*M* phosphate ten times.

(*c*) *Effect of temperature.* The rate of CO_2 output by the standard technique of Section I at 0·2° C. and 19·2° C. showed a temperature coefficient per 10° C. of 2·1 for the catalysed part of the reaction, the enzyme used being a purified $Ca_3(PO_4)_2$ preparation. Much further work is needed here also.

(*d*) *Effect of* *p*H. This has not yet been studied systematically, but the impression gained is that the enzyme has an optimum in the physiological range. For CO_2 uptake experiments above *p*H 11, and for output experiments below *p*H 5·5, it will be necessary to resort to Hartridge and Roughton's rapid reaction methods, or perhaps to some variant of the processes discussed under the heading "Scope of enzyme action" in this section.

(*e*) *Neutral salts.* High concentrations of salt, especially NaCl, have been found to exert a very depressing effect upon the enzyme activity both in CO_2 output and CO_2 uptake experiments.

(*f*) *A special advantage of carbonic anhydrase for kinetic studies.* Brinkman, Margaria and Roughton [1933] have measured the rates of the reactions $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ under the conditions where the time for the reaction to be half completed ranged from 15 min. to 0·01 sec., *i.e.* a 100,000-fold variation. These same methods are applicable to the catalysed reaction, which it is therefore hoped to follow over a much wider range of enzyme concentration than has been tried in the case of any other enzyme reaction.

Section V. The action of various inhibitors, and possible activators.

The substances which have been tried may be divided into four classes:

(*a*) Those which might act by poisoning some active centre in the enzyme of a metallic type (*e.g.* a hæmatin, iron or copper nucleus), viz. carbon monoxide (in dark and in light), cyanides, sulphides, azides, pyrophosphates.

(*b*) Other anions which have been shown to inhibit particular enzyme systems, viz. iodoacetate, fluoride.

(*c*) An extensive series of metallic salts, representative of most of the groups of the periodic table.

(*d*) Miscellaneous substances.

The general method used for testing the activity of the enzyme in presence of these various substances has been that described in Section I.

Classes (a) and (b).

Carbon monoxide. The boat technique was used with the following modification: the phosphate solution and bicarbonate solution, with enzyme contained in one or other of them, were saturated with pure CO in Thunberg tubes. Controls in which the enzyme was added subsequently showed that this shaking did not itself cause inactivation of the enzyme.

2 c.c. of each CO saturated reagent were then placed in the boat, the air in the latter replaced by pure CO, and after temperature equilibration the boat was shaken and CO_2 output followed in the ordinary way. The CO was prepared by acting on sodium formate with conc. H_2SO_4 and was stored under pressure over alkaline sodium hydrosulphite. It thus contained no O_2 or CO_2. Controls in which hydrogen was stored over alkaline sodium hydrosulphite under the same conditions showed that the gas did not thereby acquire any traces of inhibitory substances (*e.g.* SO_2, H_2S or particles of liquid spray). For experiments in the dark the water in the bath was replaced by a deeply coloured dye solution, whilst for light experiments a mirror was placed at the bottom of the bath, and a 100 watt lamp was placed just above the surface of the clean water in the bath.

The results of some experiments are given in Table II:

TABLE II.

Nature of enzyme preparation	Date	Activity expressed in p.c. of activity in air		In CO dark / In CO light
		In CO dark	In CO light	
Dilute human blood	29. iv. 32	—	—	80·0 p.c.
Crude $CHCl_3$ enzyme	16. ix. 32	36·0	32·0	112·0
$Al(OH)_3$ purified enzyme	20. iv. 32	36·0	70·0	53·0
$Ca_3(PO_4)_2$ purified enzyme	23. vi. 32	56·1	59·5	94·5
" " "	22. viii. 32	23·3	30·5	77·0
" " "	29. vi. 32	25·0	22·7	110·0

In all instances carbon monoxide produced a large inhibition in the dark; in half the cases, viz. the crude blood, the $Al(OH)_3$ preparation and one of the $Ca_3(PO_4)_2$ preparations, this inhibition was partially reversed by light, though in the other cases the light rate and the dark rate in CO were almost the same within experimental error. In none of the four purified preparations was there any visible trace of hæmatin present, but all these showed marked inhibition by CO in the dark, and in two cases this inhibition was partially reversed by light. Further work,

however, is needed to discover why the effect of light is variable, and in positive cases to investigate its action in detail.

Cyanides. The action of HCN (prepared by neutralization of KCN to *p*H 7·4) was studied upon crude blood and upon the enzyme in the various stages of purification described in Section II, methods *B* and *C*. The effect of various concentrations, ranging from *M*/800 to *M*/16,000 HCN, upon a $Ca_3(PO_4)_2$ purified enzyme solution, is shown in Fig. 5. Practically complete inhibition is produced by *M*/800 HCN. The latter

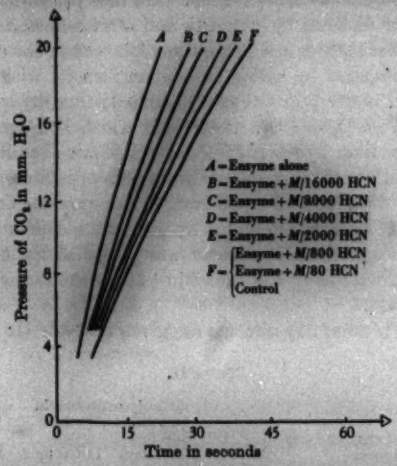

Fig. 5. Effect of various concentrations of HCN upon activity of enzyme.

was also found to be the case with four different samples of crude ox blood, one sample each of horse blood and whale blood, and one sample of enzyme purified by method *B* of Section II.

Considerable inhibition by *M*/4000 HCN was noted in the case of one sample of $Al(OH)_3$ purified enzyme, including the ultra-filtrate from the latter through a 6 p.c. Bechhold membrane, and also in the case of one sample of human blood. These two cases were not tested with *M*/800 HCN.

At the outset of our research we met with a solution of electro-dialysed ox hæmoglobin, which showed slight activation by *M*/1000 HCN instead of inhibition. Brinkman [1932] also has met with such cases.

Unfortunately we threw away all our material before we realized its value, and since then we have met with no other such case, though we have been continually on the look-out. The detailed investigation of an anomalous sample of such a kind might well throw important light on the nature of the enzyme.

Sulphides. The same group of enzyme preparations as those studied for cyanide inhibition (with the exception of human and whale bloods and the $Ca_3(PO_4)_2$ purified enzyme, none of which were tested at all) also showed complete inhibition by *M*/1000 Na_2S. At lower concentrations than *M*/1000, sulphide appears to be a somewhat more effective inhibitor than cyanide. Sodium sulphide was also shown to inhibit the catalysis of CO_2 uptake by 4·0 *M* aqueous ammonia.

Sodium azide (NaN_3). A *M*/500 solution of this reagent was tested on one sample of $Al(OH)_3$ purified enzyme and caused a partial inhibition.

Pyrophosphate, sodium fluoride (*M*/1000), *sodium iodoacetate* (*M*/1000), were all negative as regards inhibition.

Class (c). Metallic salts.

Salts of twenty-three different metals were tried, the final concentration in most cases being *M*/1000 or *M*/2000. A $Ca_3(PO_4)_2$ purified enzyme preparation was used in every case except Na, K and Mg. In the latter a crude $CHCl_3$ preparation was used. Negative results were found in the following groups of the periodic table.

Group 1 A. Na, K.
Group 2 A. Be (as sulphate), Mg (as sulphate), Ca.
Group 2 B. Cd (as sulphate and as chloride).
Group 3 A. Ce (as chloride).
Group 3 B. Al.
Group 4 A. Ti (as chloride).
Group 4 B. Pb (as nitrate).
Group 6 A. Cr (as chloride).
Group 7 A. Mn.
Group 8. Fe (as ferrous sulphate + ferric chloride), Co (as nitrate), Ni (as chloride), H_2PtCl_6.

Positive results were found for:

Group 1 B.	Cu (as 0·0005 *M* $CuSO_4$ recrystallized)	About 50 p.c. inhibition
	Ag (as 0·001 *M* nitrate)	Large inhibition
	Au (as 0·001 *M* $HAuCl_4$)	„ „
Group 2 B.	Zn (as 0·001 *M* $ZnSO_4$ not recrystallized)	About 50 p.c. inhibition
	Hg (as 0·0005 *M* $HgCl_2$ recrystallized twice)	Large inhibition
Group 5 A.	Va (as 0·001 *M* vanadyl sulphate not recrystallized)	„ „

With the exception of vanadium, metals which act as poisons to carbonic anhydrase are those of Groups 1 B and 2 B only. Such also have

been observed for a number of other enzymes, viz. xanthine oxidase [Wieland and Mitchell, 1932], saccharase [Myrbäch, 1926], papain, kathepsin [Krebs, 1930], and may indeed be an empirical principle of wide application for enzymes. The present case, however, cannot yet be quoted as a satisfactory example, because the reagents used in testing the enzyme activity, viz. phosphate and bicarbonate at *p*H 9·2, both form precipitates with many of the metals tested. Some of the inactive metals may have been ineffective on account of this purely inorganic factor, and we cannot be sure that if it were controlled there would any longer be such a sharp demarcation between them and the active group Cu, Ag, Au, Zn and Hg.

Such control would be very difficult to effect. At all events there is no doubt that Cu, Ag, Au, Zn and Hg are strong poisons for carbonic anhydrase.

Class (d). Some other substances.

Indifferent gases: N_2, O_2, H_2. The activity of the crude $CHCl_3$ enzyme in nitrogen, oxygen and hydrogen was found to be the same as in air.

Phenylurethane. This was chosen as an example of a commonly used cell narcotic. The phosphate and bicarbonate solutions were saturated with phenylurethane by shaking in Thunberg tubes for an hour or more and were then allowed to stand. Horse hæmoglobin, crude $CHCl_3$ enzyme, and $Ca_3(PO_4)_2$ purified enzyme all showed about the same inhibition, *i.e.* roughly 50 p.c.

Saponin. A 4 p.c. solution of ox blood dissolved in 1 p.c. saponin showed the same activity as a 4 p.c. solution of the same ox blood in water.

Hydrazine sulphate. A 0·002 *M* solution had no effect upon the activity of a crude $CHCl_3$ preparation.

Ferricyanide. Blood dissolved in 0·001 *M* K_3FeCy_6 has the same activity as blood dissolved in water.

Section VI. The distribution of the enzyme and some physiological considerations.

Distribution.

During this work, many estimations of the enzyme content of goat, ox and human blood have been made. These are collected in Table III, which also gives the content of some other mammalian bloods in single instances.

TABLE III.

Animal	No. of cases	*E*/c.mm. blood	Average *E*/c.mm. blood
Goat (anæsthetized)	11	1·30–6·10	2·80
Ox (defibrinated)	6	0·60–1·80	1·10
Man	8	0·37–0·68	0·55
Rabbit	2	0·71–1·72	1·21
Goat (normal)	1	1·35	1·35
Horse	1	0·64	0·64
Rat	1	1·70	1·70
Whale	1	1·40	1·40

The values were the same, whether clotting was prevented by defibrination or by oxalate. In any one species there is a two- to four-fold variation or more in normal animals; this suggests that the normal carbonic anhydrase content of mammalian blood is abundantly in excess of minimum physiological requirements. It seems doubtful whether there is any significance in the higher average content of goat's blood as compared with ox, and man.

During the last four months of this research we were lucky enough to have the opportunity, through the kind offices of Prof. Barcroft and his colleagues, of studying an exceptionally interesting case of distribution.

About a dozen female goats were "stocked" during the latter part of January 1933 and were operated on in roughly successive weeks from March 20 to June 1. Samples of the fœtal blood and maternal blood (treated with oxalate and fluoride) were given to us in each case and the carbonic anhydrase content of each estimated. The results are shown in Table IV, together with a single case of whale mother and fœtus, the blood of which was kindly supplied by Mr A. Laurie.

TABLE IV.

Date of operation	Date stocked	*E*/c.mm. mother blood	*E*/c.mm. fœtal blood	Activity of fœtal blood expressed in p.c. of activity of mother blood
Mar. 23	Feb. 2	1·6	<0·010	<0·5
April 11	Jan. 30	3·6	0·022	0·6
„ 13	„ 24	4·2	0·140	3·3
„ 18	„ 23	1·8	0·060	3·4
„ 24	„ 21	6·1	0·110	1·8
May 1	„ 20	1·3	0·067	5·2
„ 5	„ 19	1·6	0·056	3·5
„ 12	„ 18	1·9	0·066	3·5
„ 22	„ 16	1·7	0·040	2·3
„ 31	„ 12	5·0	0·456	9·2
June 1	Unknown, probably 15 weeks ±	1·9	0·292	15·0
May 31	Whale fœtus of unknown age	1·4	0·350	25·0

In the very young fœtuses there is extraordinarily little enzyme and the amount does not begin to rise appreciably till very near the end of term. Since the fœtuses live in an all fluid environment there is perhaps not so much need for the enzyme, since it is possible for them to excrete HCO_3^- into the mother blood as well as CO_2. It is, however, indispensable for them to have a good supply before parturition occurs, otherwise when they begin to breathe they may not be able to excrete CO_2 gas into their expired air fast enough. The consequences of the low content of enzyme in fœtal blood must indeed be taken fully into account in working out quantitatively the gas exchange between maternal and fœtal blood in the placenta. Finally, since the hæmoglobin content of the fœtal blood was never less than 50 p.c. of that of the maternal blood, this series of experiments gives a clarion instance of the independence of the enzyme from hæmoglobin.

The distribution of the enzyme in the blood of lower organisms, *e.g.* earthworm, planorbis, etc., was reported in the preliminary communication of Brinkman, Margaria, Meldrum and Roughton [1932]. The measurements of activity were made by a portable, but much less accurate, modification of the boat technique, and are too rough for values of E/c.mm. to be worth calculating.

Tests on some other materials besides blood have been made with the following results:

Cow's milk, normal human urine. Both inactive even when 1 part in 10 is added to the phosphate solution in the boat test.

Rabbit's sperm. Showed a content of 0·0034 E/c.mm. This sperm was kindly supplied by Dr J. Walton and appeared to be quite free from blood.

Muscle extract. Water extracts of the hind limbs both of a dog and a cat were made, after the muscles had been perfused with isotonic saline until the issuing fluid was almost free of blood hæmoglobin. In both cases an activity was observed, and it was found that the enzyme activity of the muscle extract/content in muscle hæmoglobin (measured colorimetrically) was roughly equal to the enzyme content of the blood/hæmoglobin content of the blood of the same animal.

Plasma. No carbonic anhydrase could be detected in oxalated rabbit's plasma even when tested 1 part in 10, provided that allowance was made for the very slight amount of laking of the red corpuscles which occurred before the plasma was separated. This is confirmed by the experiments with unlaked goat's blood described below.

Yeast. No enzyme could be detected in an extract of 15 g. Belgian top yeast ground with 100 c.c. of a mixture of $M/5$ Na_2HPO_4 and $M/5$

K_2HPO_4 and allowed to stand for 24 hours at 1° C. before being centrifuged.

In one experiment with a zymin preparation a slight positive effect was noticed. This needs confirmation. No carbonic anhydrase has yet been found in green plant material.

Comparison between laked and unlaked blood.

In early experiments, confirmed recently, with defibrinated ox blood, obtained from the butcher, we noticed that the activity of unlaked blood was about 23–35 p.c. of that of the same amount of laked blood. This was also true if the unlaked blood corpuscles were washed three times with saline to eliminate the slight laking which is almost always present in defibrinated blood. We attributed the lower activity of the unlaked corpuscles simply to the fact that the substrate has to diffuse into the corpuscles before it can react with the enzyme, so that this is another case of chemical reaction velocity and diffusion being joint limiting factors [Roughton, 1932].

We were, therefore, surprised to find that in goat's blood, drawn in the laboratory by syringe from a vein, the activity of unlaked corpuscle suspensions was, in all cases but one, only 0·05–0·01 p.c. of the activity of the same amount of laked blood, when tested by the method of Section I. In each case the blood was put in the 0·2 *M* phosphate, and the result was the same whether the goat was anæsthetized or not and independent of whether clotting was stopped by defibrination or by oxalate-fluoride solution.

The fact that whole blood was used for these "unlaked" experiments also confirms that plasma and serum are both practically inactive. The very slight activities observed may indeed be due to traces of laking.

The result must apparently be a permeability effect of the corpuscle membrane, which may depend upon the species of animal, the manner in which the blood is drawn, and the medium in which it is suspended. In any case, much further work on these lines is needed not only to clear up the subject for its own sake, but also to enable us to assess the activity of carbonic anhydrase in circulating blood *in vivo*.

Possible rôle of the enzyme in other processes than evolution of CO_2 *in the lung.*

Carbonic anhydrase may possibly have some effect in earlier stages of the metabolism, than the final one in which CO_2 is evolved from the body. The only one so far tested is the fermentation of glucose-phosphate

mixture *p*H 7·6 by zymin at 26° C. No effect was observed. Many other possibilities, such as decarboxylation, formation of urea in the liver, effect in muscular activity, etc., need to be explored, as do also the effects of injection of the purified enzyme. Brinkman [private communication] tells us that he has made a start at the latter problem. Further work, on such lines, is contemplated in collaboration with him and his co-workers.

Summary.

1. Carbonic anhydrase is an enzyme present in red blood corpuscles (but not in the blood plasma) which catalyses both phases of the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. It is thus of prime physiological importance in the formation of CO_2 from bicarbonate in the lung. Without it, CO_2 could not be excreted nearly fast enough for the needs of the body.

2. The activity of the enzyme can be estimated by its accelerating effect upon the evolution of CO_2 from a mixture of 0·2 *M* phosphate buffer *p*H 6·8 with 0·2 *M* bicarbonate. At low concentrations of enzyme, the acceleration is directly proportional to the amount of enzyme added.

3. The enzyme can be separated from the hæmoglobin of the red blood corpuscles by coagulating the hæmoglobin. Three different methods were tried with success. In the best of these, ox corpuscles are treated with an equal volume of 40 p.c. alcohol, and then with half their volume of chloroform. The mixture is then shaken and centrifuged. The supernatant fluid is practically colourless and contains roughly 50 p.c. of the total enzyme of the blood. This crude preparation is further purified by adsorption with $Al(OH)_3$ cream or $Ca_3(PO_4)_2$ suspension and by ultra-filtration. The purest product is about 1800 times as active as the original blood and seems to be free from hæmoglobin, hæmatin, catalase, peroxidase and oxidase. The enzyme is very stable when evaporated to a solid.

4. The enzyme is stable in solution over the range *p*H 3–12. Its solution in water is destroyed by 30 min. heating at 65° C. It shows most of the protein reactions. It accelerates a number of processes with over-all velocity dependent upon the rate of one or other of the reactions $CO_2 + H_2O \rightleftharpoons H_2CO_3$, or $CO_2 + OH^- \rightleftharpoons HCO_3^-$.

5. The enzyme is poisoned by CO, cyanides, sulphides, azides, Cu, Ag, Au, Zn, Hg, phenylurethane. A number of other substances were tested, but had no effect.

6. Preliminary work has been done on the kinetics of the enzyme action, and the effect of temperature, salts and *p*H thereon.

7. The fœtal blood of goats has far less enzyme than the maternal blood, but the content rises towards the end of gestation. A suspension of unlaked corpuscles is much less active than a solution of laked corpuscles.

8. Some other instances of the distribution of the enzyme are given.

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THE STATE OF CARBON DIOXIDE IN BLOOD.

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Introduction.

In another paper in the present *Journal* we [Meldrum and Roughton, 1933*b*] have described the isolation from blood corpuscles of the enzyme carbonic anhydrase, which catalyses both phases of the reversible reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. In the introduction to that paper and elsewhere we pointed out that it is only through the presence of this enzyme that such CO_2 as is carried as bicarbonate in the blood can be transported to the expired air fast enough for the needs of the body. If all the carbonic anhydrase is either removed from the blood, or is prevented from acting by the addition of some poison, *e.g.* cyanide, then the change from CO_2 to bicarbonate and *vice versa* must become relatively slow, and any rapid chemical processes still occurring between such "mutilated" blood and CO_2 must be due to some other chemical reaction.

We shall begin by describing the rate of CO_2 output from, and CO_2 uptake by, cyanide blood as compared with normal blood.

By such means we have confirmed the view of Henriques [1928] that, under suitable circumstances, appreciable amounts of CO_2 combine rapidly and reversibly with blood to form compounds of some other kind than bicarbonate, and to a greater extent in reduced blood than in oxygenated blood. We next give a series of experiments upon CO_2 uptake at various *p*H's by ammonia, certain amino acids and polypeptides, which parallel very closely the experiments upon cyanide treated blood, and hence lead to the conclusion that the CO_2 compound in the case of blood is of the same type as in the case of the simpler amino compounds, namely of a carbamate type. In the last section of this paper we consider the results of previous workers upon the nature of CO_2 compounds in blood in the light of this conclusion, and also discuss shortly the physiological importance of carbamates in CO_2 transport.

Section I. CO_2 output experiments on normal and on cyanide-poisoned blood.

To begin with, it will be useful to recall Henriques' [1928] experiments in some detail. Hæmoglobin solutions or serum were equilibrated with various CO_2 pressures, and 2 c.c. samples of such solutions were then introduced suddenly into the evacuated chamber of a modified van Slyke gasometric apparatus. Immediately afterwards the apparatus was shaken for a known period, at the end of which the shaker was stopped and the amount of CO_2, which had escaped into the gas phase, was measured by the usual methods. A series of such experiments

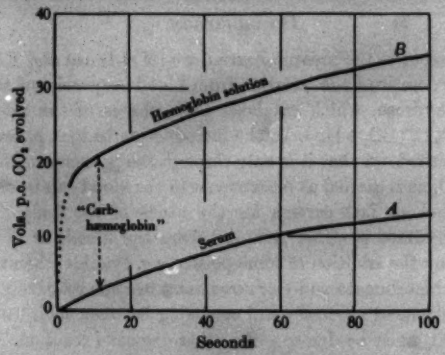

Fig. 1. Rate of evolution of CO_2 from hæmoglobin solutions and from serum as studied by Henriques.

with shaking periods of 5 sec. and upwards (to 10 min.) gave the curve of CO_2 evolution plotted against time.

In the case of horse serum the curves were of the type shown in Fig. 1, *A*, *i.e.* were similar to those of Brinkman, Margaria and Roughton [1933] for the rate of CO_2 evolution from mixtures of bicarbonate and phosphate buffer, and indeed the actual rate of CO_2 evolution from serum was of the order to be expected from the velocity constants of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ in absence of catalyst.

In the case of horse hæmoglobin solutions, however, the curves were of a different type (Fig. 1, *B*). A certain proportion (in some cases as much as 40 p.c.) of the CO_2 came off very suddenly in the first 5 sec., whereas the remainder of the CO_2 came off in the same gradual manner,

and at about the same rate as, from serum or from phosphate-bicarbonate mixtures of that *p*H. Henriques thought that the sudden rapid evolution of CO_2 could not be explained by the presence in his hæmoglobin solutions of a catalyst for the $H_2CO_3 \rightleftharpoons H_2O + CO_2$ reaction, for there seemed no conceivable reason why such a catalyst should only act for 5 sec. and then stop acting. He attributed it, on the other hand, to the rapid dissociation of a reversible compound between CO_2 and hæmoglobin, which he called "carbhæmoglobin."

These results were very mystifying to us, in view of the large amounts of carbonic anhydrase present in blood, and of the fact that hæmoglobin solutions prepared by the ordinary methods have so far proved to be richly contaminated with this enzyme.

We started then by repeating some of Henriques' experiments with a more labour-saving technique which enabled us to get the whole curve for the CO_2 evolution on one sample of solution in a single experiment:

2 or 4 c.c. of ox blood, laked by freezing and thawing, was equilibrated thoroughly with air containing 50 mm. Hg pressure of CO_2, and then introduced suddenly into a glass boat-shaped vessel (capacity 72 c.c.), mounted in a shaker in a constant temperature water bath. The boat was then at once closed by a stopper, a lead through which connected with the manometer, and the shaker started as soon as possible. From readings of the manometer at 5, 10, 15, 30 sec., etc., the curve of CO_2 evolution was plotted. This technique is thus a particular example of the "boat" method of Brinkman, Margaria and Roughton [1933], used by them in studying the kinetics of the uncatalysed $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ reaction.

The results with normal laked blood, whether of ox or horse, were always of the type shown in Fig. 2, *C*. There was no evidence of the curve consisting of two phases, one rapid, the other slow; on the other hand the CO_2 evolution proceeded smoothly to completion in about 60–90 sec. It was indeed just what we expected, for the laked blood contained abundant amounts of carbonic anhydrase, and this should have caused the CO_2 to be formed from the bicarbonate of the blood and evolved into the gas phase as fast as diffusion conditions permit. In the boat method, controls have shown that, with watery solutions, diffusion prevents the evolution of CO_2 into the gas phase being completed in less than 20 sec., no matter how fast the antecedent chemical formation of dissolved CO_2 takes place. It is not surprising that with a much more viscous medium-laked blood the time should be about thrice as long.

The only explanation of Henriques' results, which we could think of, was that in some way or other the carbonic anhydrase in his hæmoglobin solutions had got eliminated or else poisoned.

To test this idea, we repeated one experiment with the addition to the blood of 0·1 *M* HCN (prepared by neutralizing KCN with acetic acid to *p*H 7·4).

Experiments to be described later show that this amount of cyanide is needed to poison the carbonic anhydrase in whole blood completely.

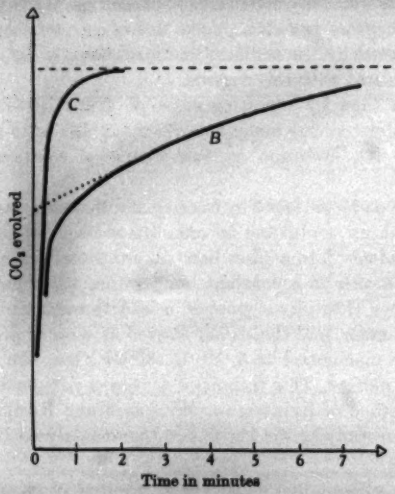

Fig. 2. Effect of poisoning carbonic anhydrase upon rate of evolution of CO_2 from blood. *B*. Blood shaken with 50 mm. CO_2, then 0·1 *M* HCN added; *C*. Blood shaken with 50 mm. CO_2, then 0·1 *M* NaCl added; then shaken with CO_2 free gas phase at 0° C.

The curve now obtained, Fig. 2, *B*, shows the typical diphasic effect of Henriques, a large part of the CO_2 coming off very rapidly and the remainder only gradually. The total amount of CO_2 evolved was very nearly the same as that evolved in a control experiment in which the blood was treated with 0·1 *M* NaCl, so as to make the ionic strength the same as in the cyanide-treated blood.

Several confirmatory experiments with slightly varying technique gave a similar result, so that our explanation of Henriques' observations is probably correct. To clinch the matter it would be necessary

to prepare horse hæmoglobin solution in exactly the same manner as Henriques did. It is hoped that it may shortly be possible to do this in conjunction with Dr Henriques.

At this stage we gave up our experiments upon CO_2 output of blood, and turned instead to the converse process, namely uptake of CO_2 by blood initially free (or almost free) of CO_2. CO_2 uptake experiments of this kind do not seem to have been done before, but have several marked advantages over the CO_2 output experiments of Henriques: (*a*) As far as the bicarbonate mechanism is concerned the rate of uptake is dependent upon the velocity of the hydration reaction $CO_2 + H_2O \rightarrow H_2CO_3$. At physiological *p*H and in absence of carbonic anhydrase the velocity of this process in the forward direction $= k'(CO_2)(H_2O)$ and, until the later stages when the back reaction becomes important, is independent of the buffer power and *p*H of the solution. It will be remembered that Hawkins and van Slyke [1930] criticized Henriques for failing to note the importance of the *p*H and buffer power of the solution in his CO_2 evolution experiments. (*b*) The hydration reaction, being one in which two molecules, viz. CO_2 and H_2O, react together, is scarcely affected by activity coefficient corrections. The dehydration velocity $H_2CO_3 \rightarrow H_2O + CO_2$, which governs the output of CO_2 in absence of carbonic anhydrase, is on the other hand very much affected by activities, for its rate is proportional to (H_2CO_3) which, in turn, is always proportional to the product of the hydrogen activity and the bicarbonate activity. There is still great uncertainty as to the value of the activity coefficient corrections in strong protein solutions. (*c*) Lastly CO_2 uptake experiments can be carried out not only at physiological *p*H's, but also at far more alkaline *p*H's (up to *p*H 11). Experiments over a wide range of *p*H have proved very valuable in determining the nature of the CO_2 compounds formed.

Section II. CO_2 uptake experiments on normal and on cyanide-poisoned blood.

Technique.

The details of the experimental technique will be described for the uptake of CO_2 by reduced laked blood poisoned with cyanide, and the small modifications for the other cases can then be treated very briefly:

(i) *Preparation of* CO_2*-free reduced laked blood.* 40 c.c. of defibrinated ox blood is mixed with 3 c.c. 0·2 *M* acetic acid [for a reason to be given later] and placed in a 1500 c.c. Büchner flask and the latter evacuated

in a water bath, temperature 30–40° C., for 1 min. The connection to the filter pump is then closed, and the flask shaken violently by hand for 1 min. Nitrogen from a commercial cylinder is then admitted to the flask, to bring the pressure up to one atmosphere, and the flask evacuated again for 1 min. and then shaken again for 1 min. The process, mentioned in the last sentence, is repeated nine times, and then repeated five times with purified nitrogen instead of commercial nitrogen. A stock of 10 litres of purified N_2 was made by storing commercial N_2 over alkaline sodium hydrosulphite, prepared according to van Slyke. The flask is left full of pure N_2 at a positive pressure, and inverted so as to seal the cork of the flask from leakage. The flask is placed in a $-10°$ C. chamber for a few hours. On thawing the flask laking is complete. Blood so treated was found on analysis to contain not more than 1–2 vols. p.c. CO_2.

(ii) *The* HCN *solution.* 1·1 *M* HCN in 1·1 *M* K-acetate *p*H 7·1–7·4 was prepared in the same manner as in Section I. 4 c.c. of this are blown by pure N_2 into the Büchner flask and mixed there thoroughly with the reduced laked blood. The dissolved O_2 carried in by the cyanide is negligible. The cyanide blood is then transferred anaerobically to a burette, closed at the top with a rubber stopper through which a lead passed to the pure N_2 stock, and having at the bottom a three-way tap.

The blood is left in the burette with a positive pressure of N_2 on top and 4 c.c. samples are drawn off for each experiment as required.

(iii) *The boat technique* (*vide* Fig. 3). The boat method was used again. One drop of octyl alcohol is placed in the boat, and the latter closed with a two-holed rubber stopper, one lead through which is temporarily closed, whilst the other is connected to the bottom of the blood burette. The side piece of the three-way tap of the burette is connected *via* a T-piece to the vacuum pump and the pure N_2 supply. The boat is evacuated and washed out with pure N_2 twice through the burette tap: the latter is then turned so that 4 c.c. of blood run into the boat and the traces of blood left in the dead spaces are blown into the boat by turning the tap so as to connect to the N_2 supply.

The boat is left with a positive pressure of N_2, its connections closed with clips C_1, C_2 and it is then placed in the shaker in the water bath.

One lead of the boat (its clip C_1 remaining closed) is connected to the manometer, whilst the other lead of the boat (C_2 also still closed) connects to a T-piece T_1. The compensating bottle B' of the boat arrangement is connected as usual by one lead to the other side of the

manometer, whilst the second lead from B' connects to another T-piece T_2.

T_1 and T_2 are connected together by a piece of rubber tubing with a clip on it (C_3). The last lead of T_2 is connected *via* a rubber tube, controlled by clip C_6, to the vacuum and pure N_2 supply, whilst the

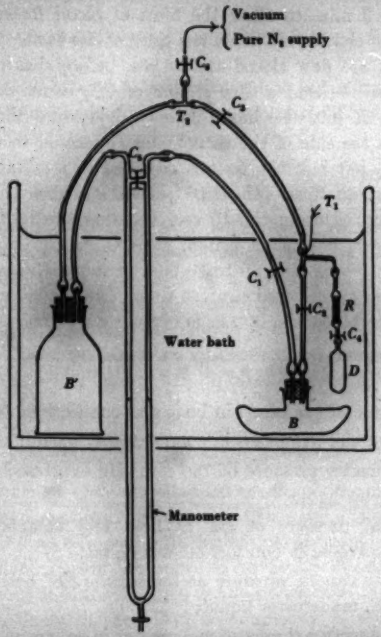

Fig. 3. Arrangement of boat, etc., for CO_2 uptake experiments.

last lead of T_1 is connected to a piece of broken thermometer tubing, R, joined by pressure tubing (with a clip C_4 on it) to a small vessel D. D has been previously filled with O_2 free CO_2, the gas being first drawn from a commercial CO_2 cylinder into a bottle containing chromous chloride, as used by Warburg, Kubowitz and Christian [1931] for removing O_2 from CO_2 atmospheres. Clip C_5 which controls the connection at the top between the two limbs of the manometer, and clip C_3

being open, the compensating bottle, manometer, and connections up to the closed clips C_1, C_2, C_4 are now evacuated and washed twice with pure N_2. Clips C_1, C_2 are now opened, and the pressure of N_2 in the whole system reduced to about 80 mm. Hg by evacuation. Clip C_6 is closed and the boat is shaken for 1 min. so as to bring the dissolved gases in the blood into equilibrium with the gas phase, after which the boat is left to rest for 5 min. to allow the films of blood formed during the shaking to drain completely down the sides of the boat.

Clips C_3, C_5 are now closed and 25 sec. before shaking is to begin clip C_4 is opened, so that a slow stream of CO_2 issues through the resistance tubing R into the boat, thereby depressing the level of the gauge liquid on the side of the manometer connected to the boat. The pressure of CO_2 put into the boat is adjusted to a suitable value, this usually taking about 15 sec., the clip C_4 closed and after 10 sec. standing, shaking is begun. During the 10 sec. standing the movement of the manometer is not more than 2 mm. at 0° C. or 15° C. Readings are taken at 5, 10, 15 sec., etc., up to 10 min. or more if necessary and the curve of CO_2 uptake plotted against time. The actual volume of CO_2 taken up by the blood, *i.e.* V c.c. is given with sufficient accuracy by the formula

$$\frac{p_0 V}{B} = \alpha x (p - W)\left(\frac{1}{B} + \frac{1}{B'}\right) + 2x,$$

where B = volume of gas phase in boat and connections to manometer;

B' = volume of gas phase in compensating bottle;

p_0 = barometer pressure of 760 mm. Hg expressed in cm. manometer liquid;

p = initial pressure of gas in boat and compensating bottle expressed in cm. manometer liquid;

W = water vapour pressure at temperature of bath expressed in cm. manometer liquid;

α = cross-sectional area of manometer in sq. cm.;

x = alteration of level of liquid on one side of manometer caused by the gas exchange (measured in cm.).

For further details, especially as to the limitations of the method, the paper of Brinkman, Margaria and Roughton [1933] should be consulted.

It is advisable in reduced blood experiments to make sure, from time to time, that no contamination with O_2 has occurred at any stage. This may be done by closing clips C_1, C_2 at the end of the shaking, and

examining the blood in the closed boat for traces of oxyhæmoglobin by the spectroscope. Such tests were invariably negative in our experiments.

(iv) *Experiments with oxygenated blood.* In this case cylinder O_2 is used instead of N_2 for freeing the blood of CO_2, and also at every other stage of the process. The evacuation and washing of the various connections, etc., need not be so scrupulous in the case of O_2 experiments as in N_2 experiments.

(v) In the case of normal blood 4 c.c. of water instead of cyanide are run into the blood.

The whole procedure sounds rather complex, but with practice an experiment with reduced blood can be done in about 35 min., and one with oxygenated blood in a few minutes less.

Experiments with normal blood.

Three experiments were done with defibrinated, laked ox blood at 0° C., and one with a solution of horse hæmoglobin prepared by washing the corpuscles three times with saline and lysing them with an equal volume of alumina cream, which on centrifuging removed the stroma proteins. The results, in all four cases, were of the type labelled normal blood in Fig. 5. There was again no evidence of two separate phases, one rapid and the other slow; CO_2 uptake proceeded smoothly to completion in 120–180 sec.

Mode of action of cyanide.

The effect of various amounts of added cyanide is shown graphically in Fig. 4. It will be seen that the curve of CO_2 uptake is slowed down, but remains smooth up to a concentration of 0·05 *M* cyanide; with 0·1 *M* cyanide, and 0·2 *M* cyanide the curve is obviously divisible into two phases. The obvious inference is that in the presence of 0·1 *M* cyanide, the carbonic anhydrase in the blood is completely poisoned, and the first rapid phase is due to the sum of the CO_2 taken up in physical solution and that taken up in some other chemical form than H_2CO_3 or HCO_3^-. The CO_2 taken up during the slow phase, however, represents the bicarbonate CO_2.

That the poisoning of the carbonic anhydrase is complete is shown by the fact that the 0·2 *M* cyanide curve is identical with the 0·1 *M* cyanide curve, except that there is a slightly greater rapid uptake in the latter case. This may, however, be explained by the lower solubility of CO_2 in 0·2 *M* CN blood than in 0·1 *M* CN blood.

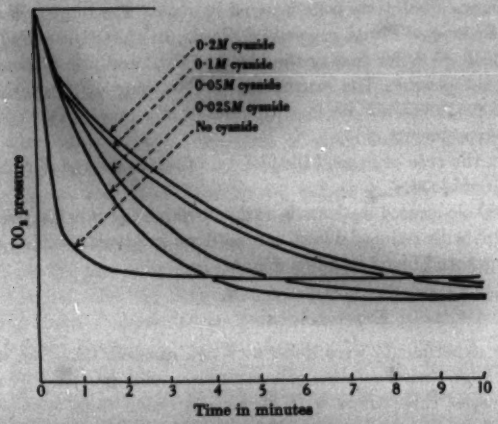

Fig. 4. Effect of various concentrations of cyanide upon rate of uptake of CO_2 by blood at 15° C.

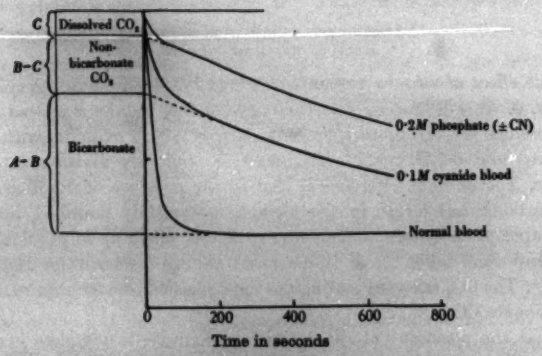

Fig. 5. Method of estimating amount of CO_2 taken up in some chemical, but non-bicarbonate form, during CO_2 uptake by blood.

That the cyanide has no important action on the blood other than the poisoning of the carbonic anhydrase is shown by the following observations and considerations:

(i) The total uptake of CO_2 by the cyanide blood is practically the same as the total uptake of CO_2 by normal blood (*vide* Fig. 4). The same is also true of the output of CO_2 from cyanide blood and normal blood in Section I. Such minor effects as are present may be attributed to difference in ionic strength or *p*H.

(ii) Fig. 5 shows the rate of uptake of CO_2 by 0·2 *M* phosphate buffer *p*H 7·6, with and without 0·1 *M* cyanide, compared with the rate of uptake of CO_2 by oxygenated blood treated with 0·1 *M* cyanide. It will be noted that the cyanide has no effect upon the rate of uptake of CO_2 by phosphate and that once the rapid phase is over in the case of the blood, the slow phase is practically identical in rate and course with the slow phase in the case of phosphate. This is strong evidence that the slow phase in the case of the blood is due to the same cause as in the case of the phosphate, namely the reaction $CO_2 + H_2O \rightarrow H_2CO_3$, followed by $H_2CO_3 \rightarrow H^+ + HCO_3^-$, with no effective catalyst present for the first of these two reactions.

(iii) As far as is known, cyanide does not act chemically upon oxyhæmoglobin or reduced hæmoglobin, though it forms a well-known compound with methæmoglobin. The matter has recently been investigated in detail by Barnard [1933].

Further evidence for this was given by a measurement of the velocity of dissociation of oxyhæmoglobin at *p*H 10, in presence and in absence of 0·1 *M* cyanide, by means of the Hartridge-Roughton [1923] rapid reaction method. The presence of the cyanide was found to have no influence.

This also makes it unlikely that the enzymes present in blood have any direct effect upon the reaction between oxygen and hæmoglobin, since the rate of dissociation, which is very sensitive to various influences, is not changed by poisoning of the enzymes of the blood.

One last point arises in connection with the cyanide. The preliminary shaking of the cyanide blood in the boat serves to get the blood and the gas phase of the boat into equilibrium as regards HCN. When the CO_2 is later taken up by the blood, part of the buffering of it must be done by the cyanide-HCN buffer system. This will give rise to some new HCN in the blood, and part of this will escape into the gas phase and be recorded as a pressure change by the manometer. It can, however, be shown by calculation that only a very slight error will ensue

The relative buffer power of the blood and the cyanide system in most of our experiments was such that about half the fresh hydrogen ions would have been buffered by the cyanide system. At worst then, every mol of CO_2 taken up could only have given rise to half a mol of HCN in solution in the blood. According to the recent data given in Landolt-Bornstein's tables the partition coefficient of HCN between gas phase and water is at low pressures of HCN at least 1 to 224 at 18° C. In blood it would be expected to be slightly less.

In most of our experiments the volume of the gas phase/volume of the liquid phase was 17·5.

Therefore for every mol of CO_2 taken up by the blood not more than $\frac{17{\cdot}5}{2 \times 224}$ mols HCN, *i.e.* 0·039 mols, could have escaped into the gas phase. Thus the error could not at most have exceeded 3·9 p.c. at 18° C., and at 0° C., owing to the increased solubility of HCN, the error must have been considerably less. It will be sufficiently (*i.e.* to within 2 p.c.) accurate therefore if the apparent value of CO_2 taken up chemically by cyanide blood at room temperature and below be increased by 2 p.c. At higher temperatures, however, a larger and more carefully worked out correction would have to be applied.

Calculation of amount of rapidly formed CO_2 *compound in cyanide blood.*

Four staple initial pressures of CO_2 were used in our experiments, viz. of 20 cm. H_2O, 40 cm. H_2O, 75 cm. H_2O and 110 cm. H_2O. Blank experiments were first done with these various pressures at 0° C. and 15° C. on 0·2 *M* phosphate buffer *p*H 7·6 containing 0·1 *M* cyanide. These were used in allowing for the amount of CO_2 taken up in physical solution during the rapid phase, assuming that the solubility of CO_2 in the phosphate buffer is the same as in blood. This assumption is almost certainly right to within 10 p.c., and since the total CO_2 taken up in the rapid phase was usually three to four times greater than that taken up in physical solution, the error from this source should not exceed 3 p.c. The mode of calculation may be illustrated by an example on reduced blood at 0° C.:

For the reduced blood, initial pressure of CO_2 at $t=0$ was	110·2 cm. H_2O
Extrapolation of the slow phase of the curve back to $t=0$ meets the pressure axis at (as in Fig. 5)	94·2 cm. H_2O
Therefore total CO_2 taken up rapidly at pressure of CO_2 is equivalent to	16 cm. H_2O
For the phosphate buffer initial pressure of CO_2 at $t=0$ was	110·9 cm. H_2O
Extrapolation of slow phase back to $t=0$ meets pressure axis at ...	104·5 cm. H_2O
Therefore CO_2 taken up in physical solution at pressure of CO_2 = 104·5 cm. H_2O is equivalent to	6·4 cm. H_2O

So CO_2 taken up in physical solution at pressure of CO_2 = 94·2 cm. H_2O would be equivalent to ... ... 5·8 cm. H_2O

Therefore CO_2 taken up in rapid chemical combination by the blood is equivalent to 16 – 5·8 ... ... = 10·2 cm. H_2O

With the aid of the calibration factor for the boat this gives an answer of 15·7 vols. p.c. CO_2 for pressure of CO_2 ... ... = 94·2 cm. H_2O

Or, allowing for HCN error, 16·0 vols. p.c. CO_2 for pressure of CO_2 ... = 69 mm. Hg.

The main lines of our argument and procedure may, for clearness, be recapitulated as follows (*vide* Fig. 5):

A. The CO_2 taken up rapidly by normal blood is the sum of
   (i) that taken up in physical solution;
   (ii) that taken up in some non-bicarbonate form;
   (iii) that taken up as bicarbonate.

B. The CO_2 taken up rapidly by cyanide blood is the sum of
   (i) that taken up in physical solution;
   (ii) that taken up in non-bicarbonate form.

C. The CO_2 taken up rapidly by a phosphate buffer is simply that taken up in physical solution.

Therefore C alone gives the physically dissolved CO_2;
B–C gives the non-bicarbonate CO_2;
A–B gives the bicarbonate CO_2;

and thus, by poisoning the carbonic anhydrase, it is possible to sort out from one another two separate reactions of CO_2, which in normal blood are indistinguishable by this technique, since in the normal case both reactions proceed rapidly.

"*Non-bicarbonate*" CO_2 *dissociation curves of oxygenated and reduced blood.*

In this manner the amount of rapidly bound non-bicarbonate CO_2 in volumes p.c. was determined at three or four different pressures of CO_2 both for oxygenated and for reduced blood at 0° C. and at 15° C. The results are given in Table I and are also plotted in Fig. 6. The curve relating "non-bicarbonate" CO_2 content to pressure of CO_2 may be called the "non-bicarbonate CO_2 dissociation curve," and is assumed to be practically the same for normal blood and for cyanide blood.

On inspection of Fig. 6 two things immediately stand out: First, reduced blood has a greater "affinity" for non-bicarbonate CO_2 than oxygenated blood has: at any given pressure of CO_2 there is 3 vols. p.c.

TABLE I.

Condition of blood	Temp.	P_{CO_2} in mm. Hg	Vols. p.c. in non-bicarbonate CO_2
Reduced	0° C.	10·6	9·8
	0° C.	22·0	11·6
	0° C.	50·0	14·0
	0° C.	69·0	16·0
Oxygenated	0° C.	11·6	40·0
	0° C.	20·4	9·1
	0° C.	63·2	11·9
Reduced	15° C.	12·0	3·4
	15° C.	23·6	5·7
	15° C.	42·5	7·2
	15° C.	65·0	9·5
Oxygenated	15° C.	12·3	1·2
	15° C.	35·4	4·9
	15° C.	65·0	4·9

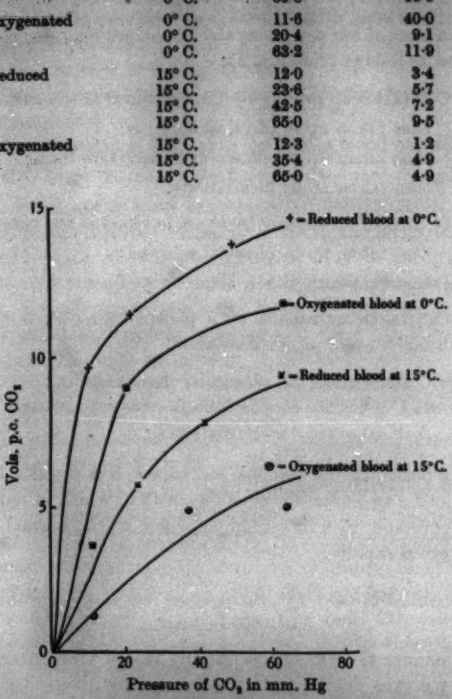

Fig. 6. Non-bicarbonate dissociation curves of oxygenated and reduced blood at 0° C. and 15° C.

or more difference between the two curves. Secondly, the "affinity" of blood for non-bicarbonate CO_2 is much reduced by rise of temperature from 0° C. to 15° C. It may also be noted that the largest amount of non-bicarbonate formation, viz. 15–16 vols. p.c. CO_2, is not much different from the O_2 capacity of blood, and is certainly appreciable in comparison

with the total amount of CO_2 carried by the blood. Further discussion of the chemical and physiological significance of the results must be reserved until the next two sections.

Section III. Carbamate compounds.

Henriques [1928] has brought forward recently the possibility that CO_2 combines directly with the blood proteins to form components of a carbamate type: it has also seemed to us very probable that the rapidly formed CO_2 compound studied in the last section is a hæmoglobin carbamate. In this section we give strong evidence in favour of this view, but before doing so it will be well to recapitulate briefly the relevant properties of carbamate compounds, in so far as these have been studied in simpler cases than the proteins. The chemistry of carbamates has been investigated very fully by Siegfried and their physical chemistry by Faurholt [1925]. It is largely to these authors that our present knowledge is due:

(i) CO_2 combines very rapidly with ammonia, amines such as amino acids, alcohols, etc., to form carbamate compounds.

In the case of ammonia the equation is

$$NH_3 + CO_2 \rightarrow NH_2COOH \text{ (carbamic acid).}$$

Carbamic acid being a relatively strong acid reacts with further ammonia to form ammonium carbamate thus:

$$NH_2COOH + NH_3 \rightarrow NH_4^+ + NH_2COO^-.$$

In the general case of an amine PNH_2 we have

$$PNH_2 + CO_2 \rightarrow PNHCOOH \rightarrow PNHCOO^- + H^+.$$

Faurholt has shown that it is only free CO_2 (and not H_2CO_3, HCO_3^- or $CO_3^=$) that can react with the amine to form the carbamate.

(ii) The heat of formation of carbamates is very high, *i.e.* of the order of 20,000 calories or more per mol CO_2: in agreement with this is the fact that they are most readily formed at 0° C., and are largely decomposed by heating to 70° C.

(iii) The calcium and barium salts of the carbamates are quite soluble in water. This gives a ready method of separating carbamates from carbonates.

(iv) The effect of *p*H upon the equilibrium between CO_2 and several amines, *i.e.* ammonia, glycine, methylamine, etc., has been carefully studied by Faurholt [1925]. It will be noted from his curves that the

percentage of chemically bound CO_2 in the form of carbamate increases very markedly as the *p*H goes from 8 to 10, and only declines beyond that because the amount of free CO_2 in solution available to take part in the equilibrium $PNH_2 + CO_2 \rightleftharpoons PNHCOOH$ becomes so minute.

In regard to (iv) we have confirmed Faurholt's conclusions by means of the boat technique. Five solutions of *p*H ranging from *p*H 8 to *p*H 10, containing in one series 0·2 *M* ammonia and in the second series 0·2 *M* glycine, were successively shaken with a standard pressure of CO_2 at 0° C.

The rate of uptake of CO_2 in the two series is plotted in Fig. 7, *A*, *B*. There is an initial rapid uptake of CO_2 complete in the first 2 min., followed by a long slow uptake.

In these simple instances there is no doubt that the rapid process represents carbamate formation, whereas the slow process represents carbonate and bicarbonate formation.

The relation between amount of carbamate (as given by the initial rapid uptake) and *p*H is shown in Fig. 8, *A*, *B*, where the titration curves of ammonia and glycine are also shown.

The parallelism between the titration curves and the amount of carbamate formation is readily explained on the assumption that CO_2 can only react with an uncharged amino group. Thus in the case of ammonia $CO_2 + NH_3 \rightarrow$ does occur, but the reaction $CO_2 + NH_4^+ \rightarrow$ does not occur, and in the case of glycine, using the modern Zwitterion theory that the neutral form is $CH_2NH_3^+COO^-$ and not CH_2NH_2COOH, the reaction $CO_2 + CH_2NH_2COO^- \rightarrow$ does occur, but the reaction $CO_2 + CH_2NH_3^+COO^-$ does not occur.

Conversely, the relation between carbamate formation and *p*H in the case of the amino acids does indeed give further evidence in favour of the Zwitterion theory. Striking illustration of this was found by comparing the results for glycine with those found in a further set of experiments, all at *p*H 8·2 (borate-phosphate buffer), on 0·2 *M* solutions of four other amino acids and peptides.

These experiments are summarized in Table II:

TABLE II.

Substance	Alkaline *pK*	p.c. in form PNH_2COO^- at *p*H 8·2	p.c. carbamate formed
Glycine	10·30	1·0	10
Glycylglycine	8·80	25·0	47
Glycylglycylglycine	8·64	36·5	56
Histidine	9·75	2·8	24
Cysteine	8·70	31·5	62

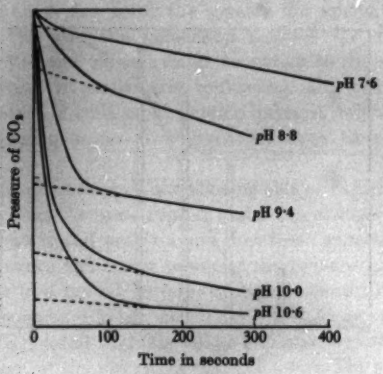

Fig. 7 *A*.

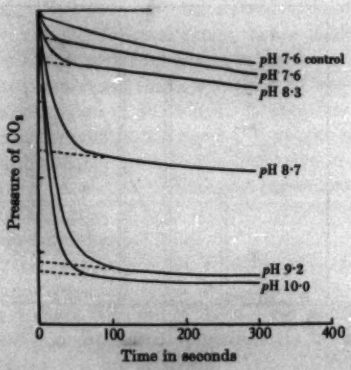

Fig. 7 *B*.

Fig. 7. *A*. Rate of uptake of CO_2 by 0·2 *M* ammonia solutions, *p*H's as shown. All the curves start from the same point, and the distance between this point and the point, where the flat part of the curve extrapolated back to $t=0$ meets the pressure axis, represents the amount of carbamate in each case. *B*. Rate of uptake of CO_2 by 0·2 *M* glycine at *p*H's shown. Intercept on pressure axis, by extrapolating flat part of curve back to $t=0$ gives amount of carbamate as in Fig. 7 *A*.

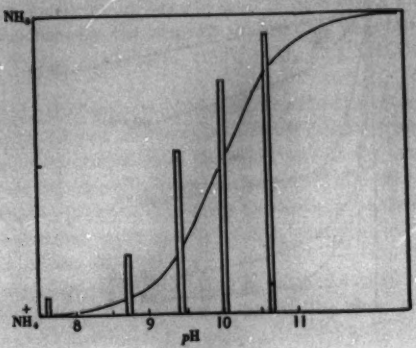

Fig. 8 *A*.

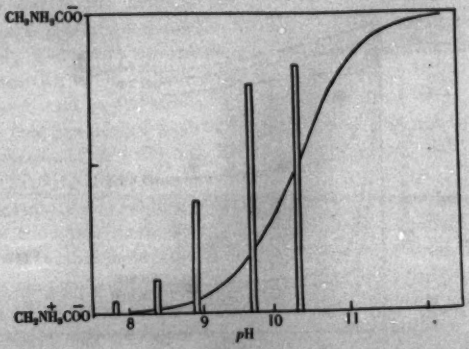

Fig. 8 *B*.

Fig. 8. *A*. Smooth curve = titration curve of ammonia. Height of rectangles = proportion of carbamate formed at corresponding *p*H. *B*. Smooth curve = titration curve of glycine. Height of rectangles = proportion of carbamino compound formed at corresponding *p*H.

It is obvious that the larger the proportion of the substance in the uncharged amino form the greater the amount of carbamate formed at given *p*H. The parallelism between the effect of peptide formation on *pK* and on carbamate formation in the series of glycine and glycylglycine is particularly convincing, and would suggest that a complex protein, if built up by peptide linkages, may well have greater carbamate forming power at a given *p*H than have its constituent amino acids.

In the case of proteins on the alkaline side of their isoelectric point, it is accepted that the same type of ionization changes occur as in the simple amino acids and peptides and that these ionizations account for the titration curve and buffer power of the proteins. At physiological *p*H the power of a protein to form carbamate should therefore depend on the relative amount in the uncharged $-NH_2$ form (as compared with that in the NH_3^+-form), and this will be indicated by the titration curve, or buffer power, of the protein in that *p*H range. The steeper the titration curve or the greater the buffer power the greater is the number of ionizations $NH_3^+ \rightarrow NH_2 + H^+$ functioning, and hence the greater the possibility of carbamate formation. It is well known from the work of van Slyke *et al.*, that hæmoglobin has a steep titration curve and a good buffer power in the physiological range: hence it should have a well-developed power of carbamate formation in that range especially at 0° C. The proteins in plasma only have one-third to one-fourth the buffer power of the hæmoglobin of the blood. It therefore seems natural to conclude that a large part of the rapid CO_2 uptake in the experiments of Section II is due in the main to the formation of carbamino-hæmoglobin compounds. A recent preliminary experiment with ox serum, rendered almost CO_2 free, showed scarcely greater rapid uptake than could be explained by physical solution.

This is further supported by a series of experiments on CO_2 uptake by cyanide poisoned blood at various *p*H's, shown in Fig. 9. The blood was treated with 0·1 *M* KCN + varying amounts of acetic acid and the *p*H's, as calculated approximately from the buffer power of blood and from the buffer power of the HCN–CN system which acts as a buffer over the range *p*H 8–10, are appended to the curves. The effect of the *p*H upon the amount of CO_2 taken up rapidly in the case of the cyanide blood is thus so very similar to the effect in the case of ammonia and glycine that it is hard to doubt that CO_2 compounds of the same kind are involved in all three cases. Control experiments with a series of similar cyanide solutions in water gave

no greater rapid uptake of CO_2 than could be accounted for by physical solution.

In preparing CO_2 free blood for the usual experiments in Section II, it will be remembered that 40 c.c. of blood was treated with 3 c.c. of 0·2 *M* acetic acid. If the latter were not added, the *p*H of the CO_2 free blood would be nearly 1·0 units more alkaline than the usual *p*H of blood, containing 40–50 vols. p.c. bound CO_2, and would therefore tend,

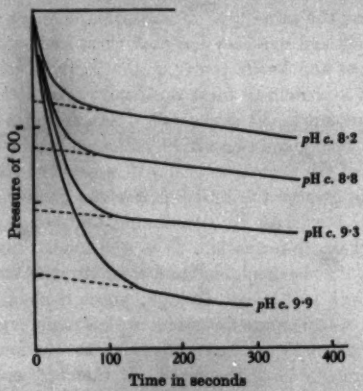

Fig. 9. Uptake of CO_2 by 0·1 *M* cyanide blood at various *p*H's. The series of curves show close parallelism with those for ammonia and glycine (*vide* Fig. 8, *A*, *B*).

as shown by Fig. 9, to take up more CO_2 as carbamate than it would do under more physiological conditions. The acid was added to counteract this effect and the amount used was equivalent to roughly two-thirds of the average amount of chemically bound CO_2 in blood, so that before shaking with CO_2 began the *p*H of the blood would only be about 0·3 *p*H more alkaline than normal, and after the rapid uptake of CO_2 is finished the *p*H would probably be quite close to normal, if it is assumed that the carbamino compound so formed is a rather strong acid.

Does the rapidly formed CO_2 compound in cyanide blood also confirm to the properties of the simpler carbamates in regard to items (i), (ii) and (iii) listed at the beginning of this section? We cannot yet

answer this question fully, but our progress so far has been quite encouraging:

(i) The rate at which CO_2 combines rapidly with cyanide-poisoned blood seems to be of the same order as in the case of its combination to form the simpler carbamates.

(ii) The heat of formation of the rapid CO_2 compound in cyanide blood should be roughly calculable from the "non-bicarbonate" CO_2 dissociation curves at 0° C. and 15° C. shown in Fig. 6, by applying the van 't Hoff isochore.

If p, p' are the pressures of CO_2 required to produce a given rapid uptake of CO_2 at the two different absolute temperatures T, T', then it might be expected that

$$\frac{Q}{R}\left(\frac{1}{T}-\frac{1}{T'}\right)=\log p/p',$$

where Q is the heat of the reaction

$$1 \text{ mol } CO_2 \text{ (gas)} + CN^- \text{ blood} \rightarrow 1 \text{ mol } CO_2 \text{ compound.}$$

This type of equation holds good for the reaction between oxygen hæmoglobin in blood.

The data of Fig. 6 only allow a rough calculation which gives a value of Q of the order of 20,000 calories. This is quite close to the value of the heat of the reaction,

viz. 1 mol CO_2(gas) + NH_3 → ammonium carbamate,

viz. 1 mol CO_2(gas) + sodium glycinate → sodium carbamino-glycinate.

These two values have been measured approximately by direct calorimetry, and the details will be given elsewhere.

It is hoped, later, to make a direct determination of the heat of the rapid reaction between CO_2 and cyanide blood.

(iii) Preliminary attempts have been made to separate carbamate compounds in blood by means of a modification of Faurholt's procedure for simpler amines. This consists in making the solution suddenly alkaline with NaOH and then mixing with excess of $BaCl_2$. The CO_2, H_2CO_3, HCO_3^- would all be turned very quickly into $CO_3^=$ which would be precipitated, but not so any preformed carbamate compounds: the latter only dissociate very slowly at alkaline *p*H's and should therefore be separable from the $BaCO_3$ precipitate by centrifuging.

In the case of blood, however, there are a number of difficulties and pitfalls. To overcome these, we have devised a special technique, which seems so far to have succeeded. The results of our first two experiments

have been positive, and indicated that in blood equilibrated with 50 mm. CO_2 there was a definite amount of CO_2 which was not precipitated at alkaline *p*H's by $BaCl_2$, and was therefore presumably in some form other than CO_2, H_2CO_3, HCO_3^- or CO_3^-. The actual amount agreed rather closely with that to be expected from the "non-bicarbonate" CO_2 dissociation curves of Fig. 6. It would, however, be undesirable and indeed unwise to go further into this matter, until the method shall have been much more fully tested.

Section IV. Relation of these results to other work on the state of CO_2 in blood, together with some physiological considerations.

The relation of previous work.

We shall not discuss any of those pre-1925 papers, which claimed to show that CO_2 combined directly with the proteins of the blood, since most of that work was gravely handicapped by the fact that the physical chemistry of proteins and of buffers, in particular those present in blood, had not been sufficiently investigated and understood. We shall just summarize and comment briefly on the more up-to-date work, and then consider it in relation to our own.

Three types of method have been used for studying the state of CO_2 in blood, namely (*a*) osmotic, (*b*) electrochemical, and (*c*) kinetic methods.

(*a*) *Osmotic methods.*

(i) Margaria [1931] found, by A. V. Hill's thermoelectric method, that the vapour pressure of blood was depressed by adding CO_2 to the blood, but not to the extent expected if the only forms present in the blood were dissolved CO_2 and bicarbonate. If, however, some of the added CO_2 combined directly with the blood proteins, or with any other compounds in the blood, such combined CO_2 would not contribute at all to depression of the vapour pressure, and hence the observed discrepancy would be explained.

(ii) Stadie and O'Brien [1931] carried out similar experiments on the depression of the freezing point of blood. Their results led them to claim, in opposition to Margaria, that the added CO_2 was osmotically active in the proportion expected from the number of mols added, and hence that no appreciable direct compounds could have been formed. Henriques [1931] has made two criticisms of these results, of which (α)

on p. 253 of his paper seems to us invalid, but the second one (β), namely that inconsistent assumptions were made as to the activity corrections, seems to us well founded.

(iii) Henriques [1933] has compared the total osmotic pressure of the interior of the red blood corpuscles with that of the serum, on the assumption that the total osmotic pressure is in each case proportional to the sum of the concentrations of all the solutes present in the two respective phases, the concentrations being measured in mols per litre of water, and the "bound" CO_2 being reckoned as 100 p.c. osmotically active. Under certain conditions he finds a greater total osmotic pressure in the corpuscles than in the serum, although he contends that there cannot, in view of the properties of the corpuscle membrane, be really any such difference. The discrepancy in the calculated osmotic pressures he attributes to the "bound" CO_2, which had been counted as fully osmotically active, but is really only in part osmotically active owing to some of it being in direct combination with proteins. The discrepancy in the total osmotic pressures he takes, indeed, as a measure of the "directly combined" CO_2.

In criticism of this ingenious procedure it must be noted that Henriques assumes that the osmotic activity coefficients of the various ions is the same in the corpuscles as in the serum. For this, as far as we know, there is no really satisfactory ground. The seriousness of this objection is shown by the fact that if the osmotic activity coefficient inside the corpuscle was only 5 p.c. less than in the serum, the whole of Henriques' calculated effect would disappear.

The uncertainty of the activity coefficient corrections in strong protein solution is indeed a bar to any rigorous demonstration of direct CO_2 compounds by osmotic methods, though it cannot be denied that strong measure of probability can be established by such means (as notably in Margaria's experiments).

(*b*) *Electrochemical methods.*

Of these the most important are:

(i) Titration of solutions of sodium hæmoglobinate with HCl and with CO_2 by Hastings, Sendroy, Murray and Heidelberger [1924], and more recently by Stadie and O'Brien [1931]. These authors obtained the same titration curve in the case of the two acids, and hence argued that there was no direct combination of either acid with hæmoglobin, or else that both combined to exactly the same extent, which latter was thought to be a most unlikely coincidence.

Henriques [1931] has, however, criticized the theoretical basis of the calculations in these experiments, and has also pointed out that if CO_2 combines directly with hæmoglobin to form a carbamino acid, this would be likely, from Faurholt's data, to be an acid with a pK near 7, and hence would tend to ionize markedly at pH near 7. This, as he points out, would enable appreciable amounts of such a compound to exist without being detected by the CO_2 titration curve.

(ii) Donnan equilibrium methods. The partition of bound CO_2 between the red blood corpuscles and serum or between a hæmoglobin solution and its dialysate, the two being separated by a collodion membrane [Henriques, 1928] fails to agree with the partition of chloride, according to the Donnan equilibrium, if all the bound CO_2 is in the form of HCO_3^-.

The discrepancy can be explained either by a different effect of hæmoglobin on the activity of the HCO_3^- ion from its effect on the activity of the Cl^- ion, or else by the supposition that some of the bound CO_2 is present, not as HCO_3^-, but in direct combination with the hæmoglobin.

(iii) The Henderson-Hasselbalch equation, viz.

$$p\mathrm{H} = pK' + \log \frac{[\text{Bound CO}]}{[\mathrm{H_2CO_3}]},$$

for the relation between pH, bound CO_2 and CO_2 pressure in blood. In reduced hæmoglobin solutions, Stadie and Hawes [1928] found that the value of pK' necessary to fit the equations was appreciably lower than in CO hæmoglobin solutions at 15° C. This they attributed to a much larger effect upon the activity coefficient of the reduced hæmoglobin than of CO hæmoglobin. The discrepancy can be much more plausibly explained, however, by supposing that a larger amount of the "bound" CO_2 was combined directly to the reduced hæmoglobin than to the CO hæmoglobin.

Margaria [1931, and in unpublished results privately communicated] has tested the Henderson-Hasselbalch equation at very low CO_2 pressures. He has observed discrepancies, which again can be explained simply by appealing to direct CO_2 hæmoglobin compounds.

It will be seen that in all these electrochemical methods there is the same difficulty, owing to uncertainty about the effect of strong protein solutions upon the activity coefficients. An opponent of the direct combination theory can always persist in maintaining that hæmoglobin has a specific effect upon the activity coefficient of the bicarbonate ion, and there is no way at present of disproving such a contention directly.

(c) *Kinetic methods, i.e.* that of Henriques, which has been described and discussed in Section I, and the method used in the present paper.

Both these methods depend upon a rather abrupt change at a certain stage in the rate of CO_2 evolution from, or uptake by, blood. It is very hard to see how the division of the rate curve into two different phases could possibly be due to any "activity" explanation, however far-fetched: we therefore conclude that in this type of experiment we have really unequivocal evidence of direct CO_2 compounds with the blood proteins. These compounds, for the reasons given in Section III, are almost certainly of a carbamate type.

It remains to compare the results of Henriques with our own. Both have found that a given pressure of CO_2 reduced hæmoglobin combines directly with more CO_2 than does oxyhæmoglobin: the difference is of the same order of magnitude in the two researches, when account is taken of the higher p.c. of hæmoglobin in Henriques' solutions. The absolute amounts of directly combined CO_2 are nearly the same in the range 40–50 mm. CO_2 pressure, but diverge above and below owing to the fact that Henriques' "non-bicarbonate" dissociation curves are S-shaped, whereas our own are more nearly hyperbolic.

If oxygenation of hæmoglobin affects the direct combination of CO_2 with hæmoglobin, then conversely the latter should affect the former, *i.e.* CO_2 should have a specific effect on the O_2 dissociation curve of hæmoglobin apart from the change of *p*H which it produces. The effect should be particularly visible at low CO_2 pressures and at low temperatures. We had intended to verify this directly, but Margaria [private communication] has informed us that he has already done so with success.

In much work on the oxyhæmoglobin dissociation curve, particularly when physiological applications have been held in mind, the blood or hæmoglobin has been equilibrated with gas mixtures containing various pressures of oxygen and CO_2. The possible existence of carbamate compounds in such cases complicates the physico-chemical interpretation of the results, and it would, indeed, from this point of view, be better to avoid the presence of CO_2 altogether. As an instance, the recent paper of Green and Root [1933] may be quoted. These authors obtained a very interesting set of dissociation curves for the blood of certain fishes at various *p*H's, calculated from the CO_2 pressure and CO_2 content of the blood by the Henderson-Hasselbalch equation. It would be satisfactory if their data could be redetermined with CO_2 free blood, the *p*H being measured by the glass electrode.

The physiological rôle of carbamate compounds.

The rôle of carbamates in CO_2 transport in the blood is probably more important in cold-blooded animals than in warm-blooded, since as shown in Section II they are so much more readily formed in appreciable amounts at low temperatures. This alternative mode of carriage, since it is a rapid one independent of enzymes, may well be of service to animals which have no carbonic anhydrase in their blood.

In order to assess the rôle of carbamates in mammalian CO_2 transport it would be necessary to have data at 37° C. Extrapolation from our "non-bicarbonate" curves at 0° C. or 15° C. suggests that at 37° C. and with physiological CO_2 pressures of 40–60 mm. Hg, reduced blood would probably contain less than 5 vols. p.c., and oxygenated blood less than 2 vols. p.c. CO_2 in direct CO_2 combination, and the difference between the two at, say, 50 mm. CO_2 might be about 3 vols. p.c. These are small figures in comparison with the average CO_2 content of blood under physiological conditions, viz. 50 vols. p.c., and the supporters of the pure bicarbonate theory may well be nearly correct on a physico-chemical basis as regards 37° C. though not as regards room temperature and below. From the physiological point of view, it is not only the nature of total CO_2 in the blood that is important, but also the cause of the difference in the CO_2 carrying power of arterial and venous blood. Christiansen, Douglas and Haldane [1914] found that, at 50 mm. CO_2, reduced blood contained about 5·5 vols. p.c. more CO_2 than oxygenated blood. If an appreciable fraction of this 5·5 vols. p.c. difference is due to direct CO_2 proteins compounds, then the latter would obviously be physiologically important in CO_2 transport. Hitherto the difference in the CO_2 dissociation curve of oxygenated and reduced blood has been attributed to oxyhæmoglobin being a stronger acid than reduced hæmoglobin. Our experiments at 0° C. and at 15° C. have, however, shown that a large fraction of the difference in the CO_2 dissociation curves of oxygenated and reduced blood at 0° C. may well be due to the direct CO_2 protein compounds, in view of the marked difference between the non-bicarbonate curves of oxygenated and reduced blood (Fig. 6).

In one case the amount of CO_2 taken up during the rapid phase by cyanide-poisoned reduced blood was 5 vols. p.c. greater than that taken up during the rapid phase by cyanide-poisoned oxygenated blood, even though the difference in total amounts of CO_2 taken up finally by the two bloods was probably not more than 7 vols. p.c. We have tried similar experiments at 37° C., but these have not yet been quite con-

vincing, for unfortunately the uncatalysed reaction $CO_2+H_2O \rightarrow H_2CO_3$ now goes so fast that it is impossible to separate the rate of CO_2 uptake into two phases, and this makes the interpretation much more difficult.

The situation, however, is far from hopeless and we hope indeed to settle the question shortly. We are anxious to do so, not only on account of the CO_2 transport problem, but also on account of the possible effects (*vide* p. 167) of these CO_2 compounds on the oxygen dissociation curve of blood *in vivo*, and of the changes which may occur in the oxygen dissociation curve after blood is drawn, if that leads to any redistribution of the forms in which CO_2 is held in the blood. The interest and importance of this matter need no explanation or justification.

Summary.

1. Henriques has claimed that when hæmoglobin solutions, containing CO_2, are shaken violently *in vacuo*, the CO_2 comes off in two distinct phases, one very rapid and the other slow and prolonged. This result could only be confirmed for whole blood if the carbonic anhydrase is prevented from acting by poisoning the blood with 0·1*M* HCN: otherwise the whole of the CO_2 comes rapidly. It is suggested that Henriques' hæmoglobin solutions contained no effective carbonic anhydrase.

2. For quantitative work the rate of uptake of CO_2 by CO_2 free blood is more suitable for study than are CO_2 output experiments of the Henriques' type.

3. Normal CO_2 free blood takes up its whole quota of CO_2 rapidly, when shaken with a gas phase containing CO_2. Cyanide-poisoned blood, however, takes up CO_2 in two phases. Of these the rapid one is due in part to physical solution but in the main to some non-bicarbonate chemical compound, whereas the second slow phase is due to the formation of bicarbonate following on the reaction $CO_2+H_2O \rightarrow H_2CO_3$, which in the present instance proceeds at about its normal rate in absence of catalyst.

4. Controls suggest that 0·1*M* HCN added to the blood has no effect of importance in the present connection other than the poisoning of carbonic anhydrase.

5. "Non-bicarbonate" dissociation curves are given for the amount of "non-bicarbonate" compound found during the rapid phase at various CO_2 pressures.

6. Reduced blood forms distinctly more "non-bicarbonate" CO_2 at a given CO_2 pressure than does oxygenated blood. Rise of temperature in both cases decreases the amount of the compound greatly, the heat of formation of the non-bicarbonate compound being of the order of 20,000 cals. per mol CO_2 bound.

7. CO_2 uptake experiments by ammonia, certain amino acids and peptides, give results so very similar to those with cyanide blood that it may be reasonably assumed that the same type of CO_2 compound is concerned in all these cases, viz. a carbamino compound. This is confirmed especially by a comparison of the effect of pH, and in a preliminary way by the magnitude of the heat of the respective reactions, and the solubility of the barium salts.

8. The place of carbamino compounds in the physical chemistry of CO_2 transport by blood is discussed in the light of previous work: so also is the question of their importance from a more physiological point of view, especially in regard to the differing CO_2 carrying powers of oxygenated and reduced blood.

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577.152:546.264]592

THE OCCURRENCE OF CARBONIC ANHYDRASE IN LOWER MARINE ANIMALS.

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(*Received August* 2, 1933.)

During the work in Cambridge on catalysis of the reaction

$$CO_2 + H_2O \rightleftarrows H_2CO_3$$

Brinkman, Margaria, Meldrum and Roughton (1932) found that although the catalyst is nearly always present in solutions of hæmoglobin, the ratio hæmoglobin/catalytic action is not constant for blood of various animals. In fish blood or frog blood the catalysis for a given amount of hæmoglobin is much less than in mammalian blood, and from the hæmoglobin-containing blood of *Planorbis* and of the earthworm the catalytic action was practically absent. These findings were explained by the discovery of Meldrum and Roughton (1932), who showed that the catalyst could be completely separated from hæmoglobin as an individual and very powerful enzyme, called carbonic anhydrase.

Nevertheless, it remained a striking fact that in the mammalian organism the carbonic anhydrase was found only in fluids or tissues where hæmoglobin was present, *i.e.* in erythrocytes and in red muscles. It might be important to know if this close relation was always maintained or whether one could find instances in which the carbonic anhydrase was present independently of the occurrence of hæmoglobin. In vertebrates the only organ in which the catalyst can be found in cells free from hæmoglobin is in the pancreas; data on this investigation will be given in a later communication.

In lower marine animals, however, the hæmoglobin and the catalyst occur quite independently, and in this paper are communicated some observations, made during a stay at the "Biologische Anstalt" at Heligoland.

[1] Rockefeller Fellow.

Methods.

The enzyme was detected by means of a simple technique, which consisted of estimating the reaction velocity between a 0·006 *N* solution of CO_2 and a 0·02 *N* solution of $NaHCO_3$ containing phenol red as an indicator. The solutions were separately contained in the stoppered upper branches of a Y-shaped tube, the lower branch being filled with mercury. Mixture of the solutions was effected by inversion of the tube; the reaction took place at a temperature between 0° C. and 1° C., the tube being placed all the time in a beaker with crushed ice. The time between inversion of the tube and attainment of the final yellow colour of the indicator was taken as a measure for the time of reaction, the average time being 75 sec. For testing a (neutralized) solution for presence of carbonic anhydrase a very small amount (0·01 c.c.) was added to the CO_2 solution in the Y-tube. Controls were always taken by adding the same amount or more of the same solution which had been warmed for 2 min. at 80° C.; they should be completely negative, that is the time for change of colour should be at least 75 sec.

The result of this investigation is expressed in the following table:

Species	Fluid examined	Presence of carbonic anhydrase	Remarks
Fucus serratus	Spermatozoa	–	
Rhodomonas	Extract of crushed cells	–	
Aurelia aurita and other types of jelly fish	Body fluid, tissue extracts	–	No Hb
Alcyonium digitatum	Tissue extract	++	,,
Actinia (*Metridium dianthus*)	,,	++	,,
Asterias	Spermatozoa	+	,,
Sea urchin	Body fluid, tissue extract	–	,,
,,	Eggs	+	,,
Aphrodite	Extract of central nervous tissue	–	Hb coloured
,,	Body fluid	–	No Hb
,,	Extract of muscles	–	,,
Nereis pelagicus	Blood	+++	Containing Hb
Amphitrite	,,	+++	,,
Arenicola	,,	+++	,,
Lumbricus terr.	,,	–	,,
Nephtys	Tissue extract	+	No Hb
Ophelia	Blood	–	Containing Hb
Harmotoë	Tissue extract	++	No Hb
Spionidæ	,,	+	,,
Nemertinæ (*Limneus*)	,,	++	,,
Buccinum	Blood	–	Containing Hcy
,,	Extract from pharynx muscles	+	Containing Hb
,,	Extract from foot muscles	+	No Hb

It is clear from this table that there is, in lower marine animals, no relation between the occurrence of hæmoglobin and of the carbonic

anhydrase. In *Alcyonium* and in Actiniæ for instance the amount of enzyme in tissue extracts is relatively large, although no hæmoglobin can be detected in these organisms. The isolation of the enzyme from Actiniæ (sea anemones, sea roses) was effected on the same lines as it was done from mammalian erythrocytes by Meldrum and Roughton. The dry, slightly yellow powder which resulted after isolation and purification could not be distinguished from the same preparation from ox blood: 1 g., dissolved in 10 c.c. of 1 p.c. NaCl solution, could be diluted $1:10^7$, and still give a definite acceleration of the hydration of CO_2. 5 min. heating at 60° C. completely destroyed this property.

In worms we find the simultaneous occurrence of hæmoglobin and carbonic anhydrase in *Arenicola*, *Nereis* and other polychætes; in *Lumbricus* and *Ophelia*, however, which contain as large amounts of hæmoglobin, the carbonic anhydrase is absent. Finally in *Harmotoë* the enzyme is distinctly present, but no hæmoglobin can be found. The presence of large amounts of carbonic anhydrase in the blood of *Spirographis* has been shown by H. M. Fox [private communication]. It was already found formerly that *Phascolosoma* (containing hæmerythrin) has a definite amount of the enzyme.

In the snail *Buccinum*, the carbonic enzyme is not present in the blood, but it can be detected in and prepared from extracts of muscles. Now the muscles of the foot are colourless, but some muscles of the pharynx are deeply red from hæmoglobin; the enzyme contents of both types of muscles are quite the same: another proof of the independent occurrence of hæmoglobin and the carbonic anhydrase.

In most of the animals and extracts investigated we have also tested for the presence of catalase and peroxydase (by the guaiacum-H_2O_2 method). There is no regularity at all in the simultaneous presence of any of the three enzymes: carbonic anhydrase, catalase and peroxydase.

Summary.

The occurrence of carbonic anhydrase in a number of lower marine organisms has been investigated. There is no correlation with the distribution either of hæmoglobin, or of catalase or peroxydase.

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612.621.8

THE GROWTH AND REGRESSION OF FOLLICLES IN THE ŒSTROUS RABBIT.

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Introduction.

In the rabbit ovulation takes place only after copulation, and, in the absence of mating, œstrus may last for months. It has usually been supposed that a single set of mature follicles persists throughout this time. Heape [1905] considered that this was usual, but not invariable, and stated that the formation of blood follicles was the normal result of regression. The absence of these structures in most normal œstrous rabbits has perhaps supported the conclusion, that mature follicles may persist for long periods in the œstrous rabbit; but, in the course of work involving successive laparotomies on the same animals, we were recently led to doubt this view. Specific experiments were then carried out, and, as a result, it appears probable that there is a continuous production and regression of ripe follicles in the unmated rabbit.

Since blood follicles are now known to be an abnormal form of follicular regression, the idea that mature follicles are short-lived in the œstrous rabbit is not incompatible with the comparative scarcity of the hæmorrhagic type.

Technique.

Œstrous rabbits isolated for at least a month previously were chosen for the experiment, and care was taken that all animals were in good condition, as it is well known that follicles may degenerate owing to dietary deficiencies.

In order to trace the life history of the larger follicles, during œstrus, it was necessary to elaborate some method of identifying them at different times and of recording their number and distribution. The idea of attempting a vital staining technique was considered and discarded, because large normal follicles are not readily permeated by vital dyes [Evans, 1916].

It was, therefore, decided to obtain a pictorial record of the ovaries at various intervals during œstrus. Photographic means were first used.

The rabbit was anæsthetized with ether and a sterile laparotomy performed. An ovary was then withdrawn, removed from its covering membrane, and orientated so that approximately the same surface area could be photographed each time. Our usual procedure was to place the fimbriæ dorsally. After the photograph had been obtained the ovary was carefully replaced, and the wound closed with catgut.

A Zeiss Greenough binocular camera was used, fixed so that it could be swung directly into position over the ovary, which was never exposed for more than 2–3 min. in all. The lighting, supplied by two 100-watt lamps, was also standardized.

In view of the unexpected results obtained, two control experiments were carried out with the photographic technique. The first consisted in photographing the same ovary twice in 30 min. from a different angle. The result (Pl. I, figs. 4*a* and *b*) shows that slight difference in the orientation of the ovary does not affect the possibility of identifying the follicles in a photograph. The second control experiment was on the effect on the follicles of exposing the ovary to the air. One ovary was photographed through the ovarian bursa, without exposure of the surface to the air, while the other was completely exposed. The subsequent behaviour of the follicles in the two ovaries was not different.

While the manipulation of the ovary for photography did not appear to be a contributory cause in the results observed, it was nevertheless decided in later experiments to reduce the time of exposure by making a rapid drawing of the ovary instead of taking a photograph. All large follicles were indicated on an outline sketch, special attention being given to the size and to the relative position of the follicles to each other and to any old corpora lutea present. This technique proved adequate for the identification of follicles and necessitated exposure of the ovary for a maximum of not more than 1 min.

With present methods it is obviously not possible to ascertain what slight effect, if any, the administration of an anæsthetic may have had.

R.F.
N.F.
1. a.
b.
N.F.
G.F.
2. a.
b.
D.F.
N.F.
R.F.
G.F.
N.F.
3. a.
b.
B.F.
D.F.
N.E
B.F.
D.E
R.F.
N.F.
4. a.
b.
5. a.
b.
6. a.
b.

Text-fig. 1.

Results.

Six œstrous rabbits were opened and photographs taken of the left and right ovaries of each. PRO 2 and 3 were photographed 27 days later, and PRO 5, 22 days later. In all ovaries the mature follicles appeared to be in different positions as compared with the first examination. It was impossible to recognize any follicle as being one of the original set. It was obvious that the mature follicles present were new and that the original follicles had disappeared completely (see Pl. I, figs. 1*a* and *b*). PRO 4 and 10 were photographed at 14 days after the first examinations. Here again the whole appearance of the ovaries had altered, no follicles being identifiable as belonging to the old group (see Pl. I, figs. 2*a* and *b*). In view of these results, another rabbit (PRO 9) was re-examined after an interval of 7 days, and it was found that the position and number of follicles present in both ovaries were markedly changed even after this comparatively short time (see Pl. I, figs. 3*a* and *b*).

This apparent difference in the mature follicles after only 7 days caused us to repeat the experiments by the more rapid technique of sketching the ovary. Seven ovaries were recorded in this manner at intervals between 7 and 27 days after the first laparotomy. The results were identical with those obtained by the photographic method (see Text-fig. I, 4*a* and *b*, 5*a* and *b*, 6*a* and *b*). After 14 and 27 days the topography of the ovaries had changed completely. After 7 days most of the large follicles had disappeared completely and a number of new follicles had developed. It then seemed necessary to examine the ovaries after shorter intervals to obtain a stage where the large follicles remained unaltered. Five additional ovaries were therefore examined at intervals between 3 and 5 days after the first inspection. The results were as follows:

Text-fig. I.

Figs. 1*a*–6*a*. Ovaries at first examination.

Figs. 1*b* and 2*b*. Ovaries 3 days after first examination. Growth of one or two new follicles, otherwise ovaries remain unchanged.

Fig. 3*b*. Ovary at 5 days after first examination. Several new follicles grown. One follicle completely disappeared and one or two possibly regressing, but general appearance of follicles unchanged.

Fig. 4*b*. Ovary at 7 days after first examination. Several follicles disappeared completely and new follicles grown; changing appearance of ovary.

Fig. 5*b*. Ovary at 14 days after first examination. Follicles have altered completely.

Fig. 6*b*. Ovary at 27 days after first examination. Similar to 14 days stage.

○ = follicle. ● = corpus luteum. *N.F.* = new follicle. *R.F.* = regressing follicle. *D.F.* = follicle disappeared completely when ovary re-examined. *B.F.* = old blood follicle.

At 3 days, the distribution and number of the mature follicles were unaltered, and except for the presence of one or two small new follicles, the ovaries were comparable in all cases with the initial drawings (see Text-fig. I, 1*a* and *b*, 2*a* and *b*).

At 5 days, the general appearance of the mature follicles was still comparable, but several small new ones had appeared. Of those previously matured, some had decreased slightly in size, while one had disappeared. In addition a follicle previously observed to be small had become mature. The remainder were easily identified as having been present in the same position as at the previous examination (see Text-fig I, 3*a* and *b*).

Since the follicular cycle is not rhythmic in the rabbit, both old and recently mature follicles are probably present at any one examination. If the entire ovary has changed after one week, the survival of mature follicles is probably of the order of 7 to 10 days. This is further indicated by one experiment in which the ovary was completely changed after 7 days, and 5 days later was again different, the largest follicles now being atretic and two or three new follicles having appeared.

Summary.

1. Mature follicles do not survive indefinitely in the œstrous rabbit.

2. There is continuous growth and regression of the follicles during the œstrous period.

3. The survival of the mature follicle seems to be from 7 to 10 days so far as can be ascertained by available methods.

The writers wish to express their gratitude to Dr A. S. Parkes for his generous help with the preparation of the manuscript and for criticisms during the course of the experiments.

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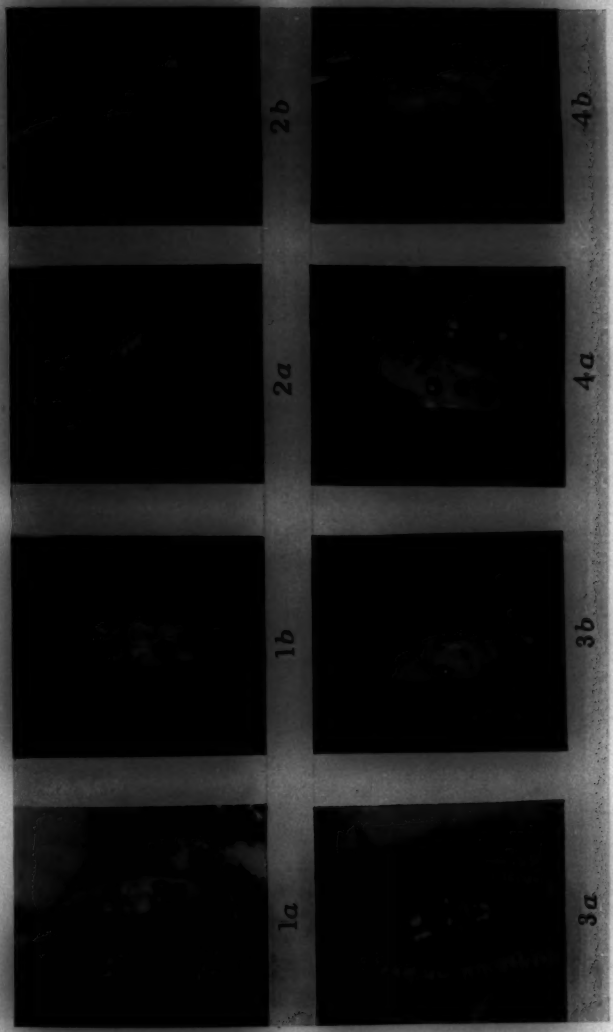
EXPLANATION OF PLATE I.

Photographs of Ovaries in situ.

Figs. 1*a*–3*a*. Ovaries at initial examinations.
Fig. 1*b*. Ovary at 27 days after first examination.
Fig. 2*b*. Ovary at 14 days after first examination.
Fig. 3*b*. Ovary at 7 days after first examination.
Fig. 4*a*. Control ovary at first examination.
Fig. 4*b*. Same ovary photographed at different angle half-hour after first examination. The follicles may be readily identified, although seen in quite a different view.



To face p. 178

612.173

THE RESPIRATORY QUOTIENT, OXYGEN CONSUMPTION AND GLYCOGEN CONTENT OF THE MAMMALIAN HEART IN AGLYCÆMIA.

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It has been shown that the respiratory quotient of the mammalian heart under hypoglycæmic conditions falls from unity to about 0·75, and this has been taken as indicating that, when the blood sugar is markedly diminished, cardiac muscle falls back on fat and protein in order to meet its energy requirements [Cruickshank and Startup, 1933]. Under the experimental conditions referred to, carbohydrate is not available because of the increase in glycogen synthesis produced by the addition of insulin to the circulating blood. Further, it has been shown that with hypoglycæmia there is a small increase in oxygen consumption and a definite and steady diminution in CO_2 production.

Further steps in this problem suggested by these results were, to investigate the gaseous metabolism of the heart under a condition of aglycæmia, to determine the lowest level to which the respiratory quotient of the heart muscle would be reduced, and to decide what is the chief source of the energy of contraction and whether or not any transformation of sugar or glycogen from protein and fat may take place.

Methods.

The respiratory quotient was determined by a method recently described [Cruickshank and Startup, 1933].

Sugar estimations were made by the Shaffer and Hartmann [1920] method in which the original procedure was modified according to details kindly supplied to us by Prof. Shaffer and since published by Murphy and Young [1932]. In the preparation of the blood-sugar filtrates, the blood proteins and the non-fermentable reducing substances were precipitated by the method of Somogyi [1930]. Lactic acid in blood and

muscle was estimated by a modification of the Fürth-Charnass method described by Lehnartz [1928]. The average percentage recovery by this method has been 99·1, with a maximal error of ± 2·9 p.c.

Glycogen was estimated by the simplified modification of Pflüger's method described by Sahyun [1931]. This method, whereby glycogen is adsorbed on purified charcoal and hydrolysed in one and the same centrifuge tube, has given excellent results. Cardiac muscle is taken with the least delay possible at the end of the experiment, cut into fairly small fragments, placed in liquid air, and 2–3 g. taken for glycogen estimation.

To make the blood sugar free, the bled dog was given insulin, 5–7 units per kg., about 1 hour before the experiment was performed. Amytal given intraperitoneally was used as the anæsthetic. During the experiment 5 units of insulin were added to the circulating blood every half hour. The attempt to render the heart glycogen free was made by adding 2 c.c. of 1 : 50,000 adrenaline every 15 min. In the experiments quoted adrenaline was added every 30 min.

The total blood volume in the isolated heart-lung preparation was carefully noted and usually was of the order of 600–800 c.c.

Results.

The results of these experiments were so remarkably uniform that after four experiments with insulin and two with adrenaline aglycæmia we proceeded with another aspect of the problem, and after two months repeated these aglycæmic experiments in order to see if this striking agreement would still obtain. It did so. The protocol published is that of the last experiment on insulin aglycæmia.

The glycogen content of the mammalian heart.

To determine the approximate correctness of our previous assumption that the glycogen content of the experimental heart is not less than 0·500 g./100 g. we carried out several experiments in which the heart was perfused with blood the original sugar content of which was not less than 0·100 g./100 c.c. Insulin was added and the amount of blood sugar disappearing and the oxygen consumption carefully noted. The sugar loss in excess of oxygen utilized was regarded as sugar stored by the heart muscle; this was subtracted from the glycogen content and the original figure thus estimated.

Table I shows by this method an average figure for the original glycogen content of 0·582 g./100 g. In section G of this table is given the

TABLE I. Effect of insulin on the glycogen content of the heart in relation to blood-sugar percentage.

Wt. of heart g.	Glycogen found g./100 g.	Blood sugar original g./100 c.c.	Glycogen added g.	Original glycogen g.	Increase p.c.
A. Normal blood sugar: isolated heart.					
50·0	0·797	0·129	0·111	0·574	38·7
76·0	0·778	0·115	0·118	0·624	24·8
60·0	0·714	0·096	0·098	0·550	17·8
			Average = 0·582 p.c.		
B. Hypoglycæmia: whole animal.					
138	0·480	—			
217	0·565	0·045			
95	0·433	0·020			
111	0·549	0·025			
87	0·640	0·036			
			Average = 0·533 g./100 g.		
C. Hypoglycæmia: isolated heart.					
Cruickshank and Shrivastava: 5 experiments, average = 0·532 g./100 g.					
D. Aglycæmia: isolated heart.					
59	0·554	Nil.			
90	0·580	Nil.			
51	0·652	Nil.			
75	0·515	Nil.			
			Average = 0·570 g./100 g.		
E. Effect of adrenaline.					
Hypoglycæmia: whole animal.					
126	0·513	0·052			
158	0·656	0·073			
			Average = 0·584 g./100 g.		
F. Aglycæmia: isolated heart.					
62	0·610	Nil.			
73	0·590	Nil.			
			Average = 0·600 g./100 g.		
G. Normal heart: glycogen percentage, whole animal.					
	0·636				
	0·513				
	0·551				
	0·650				
			Average = 0·581 g./100 g.		
Cruickshank and Shrivastava: 7 experiments, average = 0·592 g./100 g.					

average figure for the heart of the whole animal, namely, 0·581 g./100 g., which is in agreement, both with the previous result and the average, 0·592 g./100 g. These figures while a little higher, probably due to the use of Sahyun's modification, are in remarkable agreement with the average figure of 0·560 g./100 g. given by Visscher and Mulder [1930] for the unworked isolated heart.

Insulin hypoglycæmia (Table IB) would, in the whole animal, appear to be produced by the storage of sugar in the liver and skeletal muscles rather than in the heart, as indicated by an average glycogen content of

0·533 g./100 g. In the isolated heart in which the blood sugar is allowed to fall to a hypoglycæmic level (Table IC) there is no loss of glycogen, the average figure being 0·532 g./100 g. [Cruickshank and Shrivastava, 1930]. In aglycæmia, of 1–2 hours' duration, produced by insulin, there is again no loss of glycogen from the isolated heart muscle (Table ID); in fact there is a slight increase, which may be due to the synthetic action of insulin in the presence of small amounts of sugar in the blood at the commencement of the experiment. It is evident then from a consideration of such figures, the average of which is 0·565 g./100 g., that our previous assumption of the percentage glycogen content of the heart was essentially correct.

The action of adrenaline in so far as its effect on cardiac glycogen is concerned is interesting in that, in hypoglycæmia as in a prolonged aglycæmia, there is no loss of glycogen (Table IE and F).

The utilization of sugar in aglycæmia.

In our previous publication [1933] the utilization of sugar by hearts perfused with a maintained blood sugar and having a respiratory quotient of unity was shown to average 5·28 mg. per g. of heart muscle per hour. The figures for sugar utilization in Table II indicate that during the first half-hour period when sugar is still present in the blood and the respiratory quotient is unity the amount of sugar used averages 2·64 mg., that is a utilization at the rate of 5·28 mg. per g. per hour.

In subsequent periods when the respiratory quotient falls below 0·85 there is little or no utilization of sugar. The steady and rapid disappearance of sugar in relation to the fall in the R.Q. is clearly shown by the uniformity of the curves in Fig. 1. It is also evident (Table II and Fig. 1) that a similar response is occasioned by the addition to the blood of 2 c.c. of 1 : 50,000 adrenaline solution every half hour. The rate of sugar utilization during the first 30 min. when the R.Q. is unity is at the average rate of 3·60 mg., which is 7·20 mg. per g. of muscle per hour. At this rate sugar is wholly removed from the blood by the end of the first hour, by which time the R.Q. is below 0·85.

The oxygen consumption and respiratory quotient of the heart in aglycæmia.

Table II and Fig. 1 indicate that there is a slight tendency for oxygen consumption to increase as the blood sugar disappears. In the first experimental period with the R.Q. of unity the average oxygen consumption is 4·14 c.c. per g. of heart muscle per hour. It can be seen from average

TABLE II. The effect of insulin and adrenaline on the combustion of oxygen, sugar, protein and fat by the heart in aglycæmia.

INSULIN.

Date	Period 30 min.	Oxygen c.c./g.	Sugar mg./g.	Protein mg./g.	Fat mg./g.	Calories per g.	R.Q.
7. ii. 33							
Heart = 75·5 g.	1	1·94	2·57	—	—	0·0105	1·11
	2	2·05	1·65	0·77	0·40	0·0136	0·92
	3	2·07	—	—	1·00	0·0094	0·68
	4	2·05	—	—	0·95	0·0088	0·65
	5	2·05	—	—	0·90	0·0083	0·62
14. ii. 33							
Heart = 51 g.	1	2·15	2·72	—	—	1·0112	1·01
	2	2·15	2·72	—	—	0·0112	1·00
	3	2·30	1·37	0·79	0·28	0·0115	0·88
	4	2·35	—	1·91	0·26	0·0103	0·78
	5	2·44	—	—	1·19	0·0111	0·69
8. ii. 33							
Heart = 90·5 g.	1	1·98	2·33	—	—	0·0096	1·00
	2	1·98	2·33	—	—	0·0096	1·00
	3	2·06	—	0·79	0·64	0·0092	0·74
	4	1·93	—	—	0·92	0·0086	0·70
6. iii. 33							
Heart = 84 g.	1	2·15	2·89	—	—	0·0117	0·99
	2	1·99	2·42	0·01	—	0·0105	0·94
	3	2·10	—	0·04	0·85	0·0096	0·72
	4	2·01	—	—	0·98	0·0091	0·69
11. v. 33							
Heart = 59 g.	1	2·18	2·94	—	—	0·0118	1·22
	2	2·35	—	0·87	0·75	0·0101	0·74
	3	2·27	—	0·41	0·92	0·0102	0·72
	4	2·38	—	—	1·15	0·0107	0·68
	5	2·35	—	—	1·15	0·0107	0·69
	6	2·27	—	—	1·12	0·0104	0·70
	7	2·13	—	—	1·05	0·0098	0·69

ADRENALINE.

24. i. 33							
Heart = 62 g.	1	3·05	3·90	—	—	0·0167	1·07
	2	3·34	1·42	1·33	0·48	0·0157	0·84
	3	3·60	—	3·34	0·21	0·0156	0·79
	4	4·14	—	0·37	1·79	0·0168	0·71
16. xii. 32							
Heart = 84 g.	1	2·48	3·30	—	—	0·0135	1·00
	2	2·66	3·32	—	—	0·0136	0·95
	3	3·15	—	2·51	0·37	0·0137	0·78
	4	3·62	—	0·93	1·14	0·0141	0·73

Average O_2 utilization prepared from Fig. 1.

Insulin: in hypoglycæmia, R.Q. 1·00, 4·14 c.c./g./hour.
in hypoglycæmia, R.Q. 0·85, 4·30 c.c./g./hour.
in aglycæmia, R.Q. 0·80, 4·36 c.c./g./hour.
in aglycæmia, R.Q. 0·70, 4·32 c.c./g./hour.

figures calculated from Fig. 1, that as the R.Q. falls from unity to 0·70 the oxygen consumption increases from 4·14 c.c./g./hour to 4·30 c.c. for an R.Q.=0·85; 4·36 c.c. for an R.Q.=0·80; and finally to 4·32 c.c. for an R.Q.=0·70, a comparatively small change which shows how steady is the oxygen consumption of the heart in the presence of such a marked change

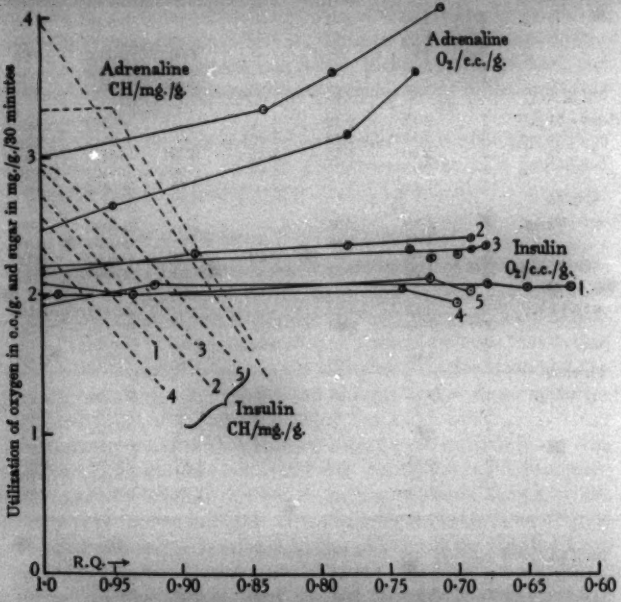

Fig. 1. Curves showing relation between R.Q.'s and the utilization of sugar and oxygen. The time between each point = 30 min.

in the type of foodstuff used for the supply of energy. The slight increase in the amount of oxygen consumed is undoubtedly due to the presence of insulin, as hypoglycæmia, *per se*, produces a slight fall in oxygen utilization.

It is also evident that neither insulin hypoglycæmia nor insulin aglycæmia produces any depression in respiratory metabolism. The addition of adrenaline occasions a marked increase in the utilization of oxygen which is accounted for first by the rapid removal of the blood sugar and secondly by the increased energy needs of the heart.

The utilization of protein by the heart in aglycæmia.

In this connection brief reference must be made to the recent revolutionary work in the chemistry of the contraction of skeletal muscle. Lundsgaard [1930–1] has shown that the iodoacetic acid poisoned muscle can contract perfectly well without lactic acid formation and with an almost complete breakdown of phosphagen. That phosphagen is broken down into creatine and inorganic phosphate in muscle activity and may thus supply for a time all the necessary energy for contraction has been demonstrated both by Lundsgaard [1930] and Lipmann and Meyerhof [1930]. Evans [1912] and others many years ago suggested that the isolated heart was able to oxidize the same materials as those used by the body as a whole, but evidence as to the possibility that protein could be burned by the isolated heart is lacking. It would appear as seen from the results in Tables I and II that when cardiac muscle shows an R.Q. of 0·80 in the absence of blood sugar and with no evidence of the utilization of glycogen, protein must of necessity be utilized. The later work of Canzanelli and Rapport [1932], who used the excess R.Q. of exercise as a criterion of the non-nitrogenous metabolism, would indicate that protein, as well as carbohydrate and fat, can be used as fuel in muscular exercise.

A survey of the possible quantities of protein utilized as shown in Table II would tend to the conclusion that there is no great call upon protein, but rather that it forms a very brief transition stage in the change-over from carbohydrate to fat. It may be argued that, since an R.Q. of 0·75 obtains when some 15 p.c. of the total oxygen may be consumed by sugar and 85 p.c. by fat, it is possible that traces of sugar may be present which the methods used do not accurately measure. But in these experiments 0·01 g./100 c.c. of sugar means that there is about 50–70 mg. of sugar available in the circulating blood, and all our experiments show that during the combustion of so small an amount of sugar the R.Q. is never below 0·85. A consideration of the periods shown in Table III indicates that when such a remnant of blood sugar has been finally removed, and if only carbohydrate and fat were utilizing oxygen, then for the R.Q. shown, the amount of sugar burned or the amount of oxygen that has been used for the combustion of the remaining sugar is in no way sufficient to meet the percentages for the distribution of the oxygen burned at the R.Q. obtaining [Zuntz and Schumburg, 1901].

For example, in the first period quoted, 70 mg. of sugar are finally burned; this demands 52·50 c.c. of oxygen. The R.Q. for the period is 0·88,

and at such an R.Q. if only carbohydrate and fat are oxidized the amount of the oxygen consumed by sugar would be 59 p.c. of the total, that is, in this period it would be 66·91 c.c. When the blood is absolutely sugar free at the end of such a period and the R.Q. is still as high as 0·88 the fact that protein may here be utilized cannot be ignored. Further, when 52·50 c.c. have been removed from the total amounts of CO_2 and O_2, the

TABLE III. Periods showing evidence of combined oxidation of sugar, protein and fat.

INSULIN.

	CO_2 c.c.	O_2 c.c.	R.Q.	Sugar used mg.	O_2 equivalent sugar used c.c.
Exp. 14. ii. 33					
Period No. 3.	104·34	118·49	0·88	70	52·50
Subtracting	52·50	52·50	1·00		
we have	51·84	65·99	0·76		
With R.Q. = 0·76					
Protein = 60 p.c.	= 31·10	38·77			
Fat = 40 p.c.	= 20·74	29·62			
	51·84	68·39		O_2 balance = −2·40 c.c.	
				S. P. F.	
				50·3 29·8 19·9	
Period No. 4.	94·16	120·21	0·78		
With R.Q. = 0·78					
Protein = 80 p.c.	= 75·33	94·16			
Fat = 20 p.c.	= 18·83	26·90			
	94·16	121·06		O_2 balance = −0·85 c.c.	
				S. P. F.	
				0 80 20	
Period No. 5.	86·53	124·84	0·69		
With R.Q. = 0·69					
Fat = 100 p.c.	= 86·53	123·61		O_2 balance = 1·23 p.c.	
				S. P. F.	
				0 0 100	
Exp. 7. ii. 33.					
Period No. 2.	142·52	154·88	0·92	126	94·50
Subtracting	94·50	94·50	1·00		
we have	48·02	60·38	0·79		
				66·3 32·0 1·7	
With R.Q. = 0·79					
Protein = 95 p.c.	= 45·62	57·02			
Fat = 5 p.c.	= 2·40	6·29			
	48·02	63·31		O_2 balance = −2·93 c.c.	
				S. P. F.	
				0 0 100	
Period No. 3.	106·89	156·97	0·68		
With R.Q. = 0·68					
Fat = 100 p.c.	= 106·89	152·70		O_2 balance = 4·27 c.c.	

TABLE III (*cont.*).

ADRENALINE.

	CO_2 c.c.	O_2 c.c.	R.Q.	Sugar used mg.	O_2 equivalent sugar used c.c.
Exp. 24. i. 33.					
Period No. 2.	173·06	207·00	0·84	88	66·00
Subtracting	66·00	66·00	1·00		
we have	107·06	141·00	0·76		
With R.Q. = 0·76					
Protein = 60 p.c.	= 64·23	80·29			
Fat = 40 p.c.	= 42·83	61·19			
	107·06	141·48		O_2 balance = −0·38 c.c.	
				S. P. F.	
				31·8 43·6 24·6	
Period No. 3.	178·15	223·56	0·79		
With R.Q. = 0·79					
Protein = 90 p.c.	= 160·33	200·41			
Fat = 10 p.c.	= 17·82	25·46			
	178·15	225·87		O_2 balance = −2·31 c.c.	
				S. P. F.	
				0 90 10	
Period No. 4.	183·24	257·60	0·71		
With R.Q. = 0·71					
Protein = 10 p.c.	= 18·32	22·90			
Fat = 90 p.c.	= 164·92	235·60			
	183·24	258·50		O_2 balance = −0·90 c.c.	
				S. P. F.	
				0 0 100	

non-carbohydrate R.Q. is in this case 0·76, which indicates that the remaining oxygen has been utilized by protein and fat for their combustion in the ratio of 60 to 40 p.c. Similar results are shown in Table III for the experiment dated 7. ii. 33.

In the following half-hour period the heart deprived of all blood sugar utilizes protein and fat in the ratio of 80 to 20 p.c. From a previous consideration of the glycogen content of these aglycæmic hearts under the influence of insulin, where it has been shown that little or no change takes place in the carbohydrate reserve of the heart, and in view of the fact that, coupled with the evidence obtained from the R.Q.'s there is a slight increase in the total nitrogen of the blood, we are further investigating the possibility that cardiac muscle in its transition from the utilization of sugar to that of fat may make use of some of the more easily oxidizable amino acids.

The possibility of the formation of glycogen from protein.

While it is argued from the results obtained here, where an R.Q. in the neighbourhood of 0·80 is maintained for at least 30 min., that protein may possibly be utilized, it cannot be demonstrated by R.Q.'s alone without further enquiry into the nitrogenous metabolism of the heart muscle. That the normal animal body can form glycogen from protein has been demonstrated by many [Atkinson, Rapport and Lusk, 1922]. The nature of the conversion depends upon the type of deamination of the amino acids, but the important point here is whether or not the conversion depends upon the presence of the liver. Mann and Magath [1922–3] maintain that the conversion is entirely a function of the liver, but results based on experiments on dehepatized animals may be misleading, and the possibility that deamination is a function of tissue in general is not yet overthrown. It is, however, impossible to arrive at any conclusion from the results here noted. All that can be said is that the rapid fall of the R.Q. to, and its retention at, 0·70 with the absence of any change in the glycogen content of the heart would indicate that while protein and carbohydrate derived therefrom may be used for fuel as suggested for the whole organism by Macleod [1928] such an event must be of a transient nature.

The changes in lactic acid in blood and heart muscle.

The lactic acid content of the heart muscle appears to vary slightly with the degree of reduction of blood sugar. The average of five normal hearts is 82·67 mg./100 g. The hearts from six hypoglycæmic bleeders gave an average of 40·40 mg./100 g., while the average for five aglycæmic experimental hearts was 41·49 mg./100 g. The average for the lactic acid content of whole blood estimated by West's method is 37·6 mg./100 c.c. for defibrinated blood; in mild hypoglycæmia it is 33·0 mg./100 c.c. In marked hypoglycæmia, the condition existing at the commencement of these experiments, where the blood sugar is not above 0·035 g./100 c.c., it is 17·8 mg./100 c.c., at the end of the first and second hour of aglycæmia the average percentage amounts are 16·30 and 10·76 respectively. In these experiments the estimation of lactic acid in aglycæmic and markedly hypoglycæmic blood was carried out by the method of Lehnartz [1928]. These figures are sufficient to show that changes in lactic acid during the course of an experiment may be regarded as negligible as far as any change in oxidative processes may be concerned.

The utilization of fat by the heart in aglycæmia.

Cardiac muscle like skeletal muscle does perform work by oxidizing preformed carbohydrate, but, as carbohydrate disappears from the circulating blood, as in hypoglycæmia *per se* or by the injection of insulin, there is definite evidence that carbohydrate no longer forms the sole fuel of activity [Cruickshank and Startup, 1933]. The question then arises, what is the final source of energy in the heart supplied with sugar-free blood? Is it preformed carbohydrate, protein, or fat? That the preformed carbohydrate supplies of the heart are not utilized has already been noted, and the possibilities with regard to protein utilization have been discussed. We are left then to discuss the evidence concerning fat combustion.

A considerable difference of opinion has arisen on the question of whether or not mammalian tissue can directly oxidize fat. Leathes [1906] and Winfield [1915] could find no evidence for the consumption of fat by normal muscle. Palazzolo [1912] found a great diminution in the fat content of stimulated muscles of both the frog and the hedgehog. Such experiments do not settle the question of direct oxidation, nor do they form evidence that the liver is the sole site for the desaturation of fats. The only evidence for direct oxidation of fat by muscle tissue comes from Lafon [1906]; it is a piece of evidence which, it would appear, has never been confirmed nor contradicted.

Few observations exist on the R.Q. of isolated skeletal muscle. Meyerhof and Himwich [1924] concluded, from experiments on the diaphragm of the rat, that the R.Q.'s were not dependent on previous diet, and, since they averaged 0·95, that sugar was the chief source of energy. Takane [1926], using a similar preparation, concluded that fat as well as protein and carbohydrate were oxidized by the muscle. We have already shown [1933] that the oxidative metabolism of the heart is exclusively carbohydrate in the presence of available sugar. This is in agreement with the results of the numerous investigations of Meyerhof, Hill and their collaborators on isolated muscle, and of Bornstein [1929] and Corkill, Dale and Marks [1930] for the eviscerated preparation. Numerous experiments upon the R.Q. of resting [Himwich and Castle, 1927] and on the contracting muscle [Himwich and Rose, 1929] would indicate that the R.Q. of normal muscle is that of the whole body, which would imply the oxidation within the muscle of a considerable amount of fat, whether directly or indirectly is an open question. That fat is utilized in exercise has been unquestionably demonstrated by

TABLE IV. Tabular protocol of one experiment.

Date: 11. v. 33. Bar. = 761 mm. Hg.
Wt. of dog = 11·5 kg. Temp. = 20·75° C.
Wt. of heart = 59·0 g. Factor = 0·912.
Heart glycogen = 0·544 g./100 g.
0·554 g./100 g.

Period 30 min.	CO_2 c.c.	O_2 c.c.	R.Q.	Heart rate beats per min.	Alveolar blood-pressure mm. Hg.	Blood in reservoir c.c.	Remarks
1	147·61	130·05	1·140	150	85	460	
2	101·80	138·43	0·735	160	90	450	Insulin 5 units 10 c.c. blood taken
3	96·71	134·25	0·720	162	90	440	Insulin 5 units 10 c.c. blood taken
4	96·71	140·53	0·688	170	98	440	
5	96·71	138·43	0·690	160	96	435	Insulin 5 units
6	94·16	134·25	0·701	164	94	435	
7	88·08	125·86	0·698	162	92	430	Insulin 5 units

First Period. R.Q. = 1·140.

Blood sugar: begin. = 0·026 g./100 c.c.; end = 0·006 g./100 c.c.

Blood sugar used = 0·170 g. O_2 = 130·05 c.c. ≡ 0·174 g. sugar.

	CO_2 c.c.	O_2 c.c.	
	147·61	130·05	
Second Period. R.Q. = 0·735.			
Blood sugar: begin. = 0·006 g./100 c.c.; end = nil.			
	101·80	138·43	
From CO_2: Protein = 35 p.c. =	35·63	R.Q. = 0·8 = 44·54	
Fat = 65 p.c. =	66·17	R.Q. = 0·7 = 94·53	
	101·80	139·07	O_2 = −6·64 c.c.
Third Period. R.Q. = 0·720.			
	96·71	134·25	
From CO_2: Protein = 20 p.c. =	19·34	R.Q. = 0·8 = 24·7	
Fat = 80 p.c. =	77·37	R.Q. = 0·7 = 110·53	
		135·23	O_2 = −0·45 c.c.
Fourth Period. R.Q. = 0·688.			
	96·71	140·53	
From CO_2: Fat = 100 p.c. =	96·71	R.Q. = 0·7 = 138·15	O_2 = +2·38 c.c.
Fifth Period. R.Q. = 0·690.			
	96·71	138·43	
From CO_2: Fat = 100 p.c. =	96·71	R.Q. = 0·7 = 138·15	O_2 = +0·28 c.c.
Sixth Period. R.Q. = 0·701.			
	94·16	134·25	
From CO_2: Fat = 100 p.c. =	94·16	R.Q. = 0·7 134·90	O_2 = −0·65 c.c.
Seventh Period. R.Q. = 0·698.			
	88·08	125·86	
From CO_2: Fat = 100 p.c. =	88·08	R.Q. = 0·7 = 125·83	O_2 = +0·03 c.c.

Zuntz [1911] and by Anderson and Lusk [1917], the latter workers obtaining R.Q.'s in the fasting dog of 0·71–0·73. The generally accepted conclusion from the work of Hill, Long and Lupton [1924] is that in mild exercise carbohydrate is used, but, that as the exercise is increased or becomes prolonged or more violent fat is drawn upon to supply the needed sugar, with a resultant fall in the R.Q. On the other hand Rapport and Ralli [1928], from the results of similar experiments on dogs, concluded that even in mild exercise the animal oxidized practically the same percentage of fat and carbohydrate as at rest, and that muscles, like other tissues in the body, oxidized carbohydrate or fat, or both, depending upon the proportions in which these substances were presented to them in available form [Rapport, 1929].

It is clear, from the evidence derived from R.Q.'s, that normal muscle can and does utilize fat. There is no doubt that this fact holds good for cardiac muscle. When a heart maintains a rate of 160 beats per min., a blood-pressure of 90–100 mm. Hg for 3 hours with not a trace of blood sugar, and an R.Q. varying between 0·72 and 0·68 with an average over five 30-min. periods of 0·699 (Table IV), then there is no question of the ability of the isolated heart muscle to utilize fat. Such results as are shown in Tables II, III and IV clearly demonstrate this fact, as well as the fact that cardiac muscle in the absence of the liver can, by desaturation, prepare the fat for oxidation. Whether it is an exclusive oxidation of fat or a transformation of fat to carbohydrate cannot be determined by the R.Q.'s alone. A further discussion of this question will form part of a subsequent paper on the changes in the R.Q. of the diabetic heart.

Summary.

The glycogen content of the mammalian heart under a condition of strict aglycæmia has been determined, and it has been shown that little or no change is effected in the carbohydrate stores in the presence of insulin or adrenaline aglycæmia.

In the production of aglycæmia the heart muscle utilizes available blood sugar at the rate of 5·28 mg. per g. of heart muscle per hour.

The oxygen consumption of the heart in aglycæmia varies from 4·14 to 4·36 c.c. per g. of muscle per hour, showing that with a fall in the R.Q. from unity to 0·70 there is but little change from the normal oxygen utilization.

With a rapid disappearance of blood sugar the R.Q. falls within 30–60 min. to 0·70, the rate of fall depending upon the amount of sugar left in the blood at the commencement of the experiment.

The R.Q. of cardiac muscle falls from unity when the blood-sugar percentage is less than 0·03 g./100 c.c. There is evidence from the R.Q. that for a short period during and after the disappearance of blood sugar, protein is being utilized, and that such a period is a brief transitional stage from the utilization of carbohydrate to that of fat.

An R.Q. of 0·70 maintained for two or more hours under aglycæmic conditions is accepted as evidence that the heart muscle resorts quickly and finally to fat as the source of its energy.

It has been demonstrated that neither insulin nor adrenaline will effect a diminution, far less a depletion, of the glycogen content of the mammalian heart.

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RELATION OF THE PITUITARY GLAND TO THE ACTION OF INSULIN AND ADRENALINE.

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Introduction.

That the pituitary gland plays an important rôle in carbohydrate metabolism has been suspected for a number of years. In 1911 Cushing observed, both clinically and experimentally, that pituitary deficiency was accompanied by an increased sugar tolerance, and references which he quotes indicate that such a relation had been suggested at an even earlier date. Very little further progress was made until 1925, when Houssay and his collaborators embarked upon detailed investigations, which have thrown much light on the problem and have led to some striking results. It is not possible here to review the large number of papers which have appeared both in the *Compte rendu de la Société de Biologie*, and in the *Revista de la Sociedad Argentina de Biología* under the name of Houssay and his associates, but the results have been very well summarized in several papers which are more easily accessible [Houssay, 1931, 1932].

In short, Houssay has come to the following conclusions:

(1) That the pituitary gland is mainly responsible for the new formation of sugar from non-carbohydrate sources—in particular from endogenous protein, and possibly also from fat.

(2) In phlorrhizin and pancreatic diabetes, where gluconeogenesis is thought to proceed at a greatly increased rate, removal of the pituitary gland leads to the suppression of this excessive new formation of sugar, with consequent reduction of the glycosuria and conservation of body protein. So striking is this effect that, in the case of the totally depan-

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creatized dog, practically all the symptoms of diabetes disappear after removal of the pituitary [Houssay and Biasotti, 1930].

(3) The diminution in endogenous protein metabolism under the above conditions is indicated by a fall in creatinine elimination, and in fat metabolism, by diminished production of acetone bodies.

(4) Converse changes are brought about by excessive pituitary action. Thus repeated injection of a suitable extract of anterior lobe produces a condition of hyperglycæmia and acidosis very similar to pancreatic diabetes.

In this connection, Houssay has presented a good deal of evidence to show that the anterior lobe is the part of the gland immediately concerned in these effects, while Geiling and his colleagues consider the posterior lobe to be responsible. Cushing first thought that the posterior lobe was responsible, but later attributed the effects to the anterior lobe.

(5) In harmony with the observations just cited on the suppression of pancreatic and phlorrhizin diabetes, removal of the pituitary body leads also to a hypersensitiveness to insulin. Thus a small dose of insulin, which in the normal animal would entail only a transient hypoglycæmia, produces in the absence of the pituitary body a progressive fall in blood sugar to a convulsive level with sometimes complete failure to rise again to the normal level. This latter characteristic has been particularly noted by Geiling *et al.* [1927] in dogs.

The explanation of this exaggerated response to insulin on the basis of Houssay's hypothesis would offer little difficulty, if we could assume that the restoration of the blood sugar to the normal level after insulin hypoglycæmia was normally accomplished by an increase in the rate of sugar production from protein and fat through the mediation of the pituitary gland. Removal of this gland, however, is known to produce profound degenerative changes in the thyroid and adrenal glands, and these glands in turn are known to be intimately connected with the maintenance of the normal blood sugar level, through the regulation of the discharge from the liver of glycogen already stored there. It therefore seemed to us worth while to investigate the relation between the increased sensitivity to insulin and the changes in the thyroid and adrenal glands, before proceeding to the more fundamental question of new formation of carbohydrate. The present paper deals mainly with the part played by adrenaline and the adrenal medulla. In future communications it is hoped to extend the investigation to the adrenal cortex and to the thyroid gland.

The evidence concerning the part played by the pituitary body in carbohydrate metabolism has hitherto been derived chiefly from experiments on dogs and a few on monkeys [Hartman, Firor and Geiling, 1930]. Although the rabbit is the species for which by far the most complete data are available as to response to insulin and adrenaline, no experiments have yet been recorded as to the effect on the response of removing the pituitary body. A method of carrying out this operation in the rabbit being now available, it seemed desirable to investigate how far the striking effects produced in the dog are to be observed also in the species from which so much of the detailed knowledge of the mechanism of carbohydrate control has been derived.

Methods.

Rabbits were used in all experiments. Pituitary removal was carried out by one of us [White] by a buccal approach described by Smith and White [1931] and in greater detail by White [1933]. All the animals were finally killed, and the endocrine glands weighed and examined histologically. Pituitary ablation was checked by serial sections of the contents of the sella turcica and in the more critical experiments by serial sections of the whole pituitary region. In most cases samples of liver and muscle were taken for determination of glycogen by the Evans' *et al.* [1931] modification of Pflüger's method. In some cases, also, extracts were prepared from one or both adrenal glands and the adrenaline content determined colorimetrically. We are greatly indebted to Dr H. Schild for kindly undertaking this part of the work.

Determinations of blood sugar were made by the Hagedorn-Jensen method. The course of the blood-sugar curve has been studied following subcutaneous injection of insulin and of adrenaline, and oral administration of glucose. Unless otherwise stated, the hypophysectomized animals were deprived of food for only 12 hours before an experiment, in view of the danger of a fatal hypoglycæmia developing during more prolonged fasting. We have further investigated the effect of pituitary removal upon the deposition of liver glycogen in young rabbits treated with insulin, following an observation originally made by Goldblatt [1929] and later extended by one of us [Corkill, 1930]. Unfortunately young rabbits survive hypophysectomy very poorly. In addition to their general lack of resistance, they have only a thin *diaphragma sellæ* and an imperfectly formed bony capsule, which in the adult almost completely separates the pituitary body from the brain. For these reasons

varying amounts of cerebrospinal fluid generally escape when complete ablation is attempted in very small rabbits. We have found that the loss of any considerable quantity of cerebrospinal fluid is always fatal. Nevertheless, in a few cases the technique has been successful. The number of such experiments is small, but the few results we have obtained are quite definite.

The influence of hypophysectomy on insulin action.

Houssay's experiments have shown clearly that hypophysectomized dogs develop a greatly increased sensitivity to the hypoglycæmic action of insulin. This statement we can fully confirm from our observations on rabbits and, further, in agreement with Houssay, we have found that hypophysectomized animals show a definite tendency to develop spontaneous hypoglycæmia. This complication has been the greatest danger in the present series of experiments. The rabbits quickly recovered from operative shock, but it was not an uncommon experience to find animals in profound hypoglycæmia despite the fact that food had not been withdrawn. In cases where liver glycogen studies were required we had to impose a preliminary period of fasting, but found that it was unsafe to extend this period beyond 12 hours. Even then, we lost some rabbits on which preliminary tests had been carried out. In animals developing spontaneous hypoglycæmia, from which we managed to obtain samples just at the pre-moribund stage, we invariably found a greatly depleted liver glycogen content.

As a starting point we decided to investigate the effect of hypophysectomy on the responses elicited by insulin, adrenaline and glucose administration. For this purpose we tabulated the responses to these substances in a series of twelve normal rabbits. At a later stage the animals were hypophysectomized and, at an interval varying from 10 to 14 days, the tests were repeated. In the case of normal animals two or three tests were carried out with insulin, adrenaline and glucose administration. The results were sufficiently uniform to justify a series of similar tests after operation. Although we could carry out repeated tests with glucose and adrenaline administration in hypophysectomized rabbits, we soon found that this was quite impossible with insulin. These operated animals soon developed an extraordinary sensitivity to insulin, and, when once hypoglycæmic symptoms had developed, it was practically impossible to avert a fatal issue.

The following experiments on Rabbit R 3 will illustrate the behaviour of most completely hypophysectomized rabbits:

Rabbit R 3. Wt. 3 kg. Injection in each case 0·5 unit insulin.

Before hypophysectomy (24 hr. fast): Initial blood sugar 98 mg. p.c. Blood sugar at hourly intervals after insulin—74, 86, 104, 114, 111 mg. p.c.

After hypophysectomy (12 hr. fast): Initial blood sugar 98 mg. p.c. 1 hr. after insulin—33 mg. p.c.; severe hypoglycæmic convulsion.

It will be observed that, before hypophysectomy, 0·5 unit of insulin produced a mild hypoglycæmic reaction from which the animal rapidly recovered, so that the effect had completely disappeared within 3 hours of the insulin injection. After hypophysectomy, on the other hand, a severe hypoglycæmia had developed within an hour of the insulin injection; the ensuing convulsions, although temporarily relieved by an intravenous injection of 1·25 g. glucose, returned within an hour and were again relieved by a second injection of glucose. An hour later the convulsions recurred with greater severity, and were no longer relieved by glucose injection. The animal was therefore killed and samples of liver taken for glycogen estimation. From our earlier experiences with animals developing spontaneous hypoglycæmia, we rather expected to find a greatly depleted liver glycogen in R 3. To our great surprise the value found was 1·6 p.c. It is important to note that a small, usually non-convulsant, dose of insulin produced a rapid and severe hypoglycæmia from which it was impossible to restore the animal by glucose administration. It is common experience that hypoglycæmia produced by much larger doses of insulin in normal rabbits can be readily relieved by glucose or adrenaline administration.

From the behaviour of R 3 and of other operated rabbits, which showed after death liver glycogen values of 2·8 and even 6 p.c., we can state definitely that the increased sensitivity to insulin is not due to depletion of liver glycogen. We have also observed a striking hypersensitivity to insulin in young hypophysectomized rabbits (see Table II). In this experiment a normal rabbit fasting 24 hours was given 1 unit of insulin and became convulsed 2½ hours later. A hypophysectomized litter mate received 0·2 unit and became convulsed 1½ hours later.

Owing to the normally wide variations found in adult rabbits, we cannot state that there is a clearly significant difference between glycogen values found in normal and in hypophysectomized animals, except in the case of the low glycogen content found in the livers of operated rabbits which develop spontaneous hypoglycæmia. Although, however, the fasting values for liver glycogen in hypophysectomized rabbits fall

within normal limits, the liver weight is greatly decreased [White, 1933], so that there is obviously less total liver glycogen present in the operated animals. Nevertheless, from actual calculations, we are certain that the total glycogen available, although on the average it is lower, is not reduced to anything like the extent found in those animals which developed spontaneous hypoglycæmia. When, in addition, glucose is given to the animal by mouth shortly before the insulin injection, we may be reasonably certain that the liver contains an adequate amount of glycogen. Even under these conditions, however, a small dose of insulin may produce a severe hypoglycæmia, as illustrated by the following experiment:

Rabbit R 9. Wt. about 2·5 kg. Injection in each case 0·5 unit insulin.

Before hypophysectomy (24 hr. fast): Initial blood sugar 120 mg. p.c. Blood sugar at hourly intervals after insulin—77, 62, 69, 65, 110 mg. p.c.

28 days after hypophysectomy (12 hr. fast): Initial blood sugar 80 mg. p.c., 5 g. glucose in 20 c.c. water was given by mouth. 1 hour later the blood sugar had risen to 143 mg. p.c. and in another hour and a half had fallen to 124 mg. p.c., at which point the insulin was injected. One hour later the blood sugar had fallen to 63 mg. p.c., and in another half hour to 32 mg. p.c., with incidence of severe convulsions. The animal was killed, and the liver glycogen content found to be 2 p.c. Autopsy showed a complete pituitary removal.

The animal deprived of its pituitary body, even when given glucose, so that the liver contained plenty of glycogen, was thus much more sensitive to insulin than when tested in the fasting condition before the operation. The fact that some animals tended to develop spontaneous hypoglycæmic symptoms, while others were apparently not so susceptible to this complication though still showing a greatly increased sensitivity to insulin, could not be attributed to variations in the completeness of pituitary removal, since animals with complete hypophysectomy, verified by serial sections of the contents of the sella, were found in both groups.

The effect of vaso-pressin and of adrenaline in relieving insulin hypoglycæmia in hypophysectomized rabbits.

The failure of glucose to effect more than a temporary restoration of hypophysectomized animals from insulin hyperglycæmia led us to investigate the action of other substances, such as vaso-pressin and adrenaline. Burn [1928] considers that vaso-pressin and not oxytocin is effective in combating insulin hypoglycæmia. The results shown below do not indicate that vaso-pressin is at all an effective agent in the animals under consideration.

Rabbit R 2. Wt. about 3 kg. Completely hypophysectomized. Fasting 12 hours.
10.50: 0·5 unit insulin.
11.35: Severe convulsion, given 50 units vaso-pressin subcutaneously. The hypoglycæmic symptoms were slightly relieved.
12.25: Severe convulsions—with slight recovery.
12.30: 40 units vaso-pressin subcutaneously—no relief of symptoms.
12.50: No abatement in severity of convulsions. Animal killed. Liver glycogen 2·86 p.c.

Apart from the failure of vaso-pressin to relieve the convulsions, this experiment is remarkable for the high glycogen content of the liver, even after a protracted insulin hypoglycæmia.

Our results with adrenaline showed a certain inconclusiveness. Even in some normal animals, adrenaline proved definitely inferior to glucose as an agent for relieving hypoglycæmic symptoms. In some hypophysectomized animals it failed to relieve symptoms, while in others it produced a slow recovery. On the whole, however, it was less effective in relieving hypoglycæmia in the operated than in otherwise normal animals. It seemed further desirable to test its effectiveness in the hypophysectomized rabbit in which we had ensured an adequate supply of liver glycogen by previous glucose feeding. The results of such an experiment, with a control on a normal rabbit for comparison, are shown below:

Rabbit 15 *a*. Wt. about 2·5 kg. Normal animal, fasting 24 hours.
Initial blood sugar 117 mg. p.c. Given 5 g. glucose orally at 9.30.
11.0: Blood sugar was 152.
12.30: Blood sugar 118; 10 units insulin subcutaneously.
1.30: Blood sugar 54; 0·5 mg. adrenaline subcutaneously.
1.55: Blood sugar 70.
2.15: Blood sugar 70.

It will be noted in this glucose-fed, normal rabbit that 0·5 mg. adrenaline checked within 25 min. the effect produced by 10 units of insulin. These normal glucose-fed rabbits were found to require at least 6–10 units of insulin to produce a definite hypoglycæmia. This is in marked contrast to hypophysectomized ones, which, despite glucose feeding, still show a severe reaction to 0·5 unit insulin.

Rabbit R 25. Wt. 2·2 kg. Operated upon 25 days previously. Fasting 12 hours.
10.30: Blood sugar 88 mg. p.c.; given 5 g. glucose orally.
11.30: Blood sugar 153.
1.30: Blood sugar 104; 0·5 unit insulin subcutaneously.
3.00: Blood sugar 25; at this point severe convulsions, which continued unrelieved by an injection of 0·5 mg. adrenaline.
The animal was finally restored by an intravenous injection of glucose.

In several other similar experiments adrenaline produced a slow but definite relief, though, as previously stated, its action was never as potent as that observed in most normal animals.

Histological observations by one of us [White] have demonstrated that hypophysectomized rabbits develop a marked atrophy of the adrenal cortex. The medulla, however, appears normal in structure and exhibits the typical chromophile reaction. Some adrenaline-content determinations on normal and hypophysectomized rabbits were kindly carried out for us by Dr Schild. Colorimetric and physiological assays were made. These results are shown in Table I.

TABLE I.

	Body wt. in kg.	Wt. of both suprarenals in mg.	Total adrenaline content in γ	Wt. of liver in g.	Liver glycogen p.c.
Completely hypophysectomized rabbits	2·9	130	< 80	42	1·34
	3·0	206	122	62	12
	3·3	205	55	65	11
	2·2	200	< 40	—	0·04
	4·2	220	110	—	—
Partially hypophysectomized rabbits	2·4	440	240	—	—
	3·8	278	160	—	10
Normal rabbits	3·1	405	85	—	—
	2·0	162	50	—	—
	3·1	522	63	—	—

There seems to be a wide variation among normal and hypophysectomized animals, and no consistent distinction between them; the results do not give any evidence of adrenaline insufficiency following hypophysectomy.

GLUCOSE TOLERANCE AND ADRENALINE HYPERGLYCÆMIA IN HYPOPHYSECTOMIZED RABBITS.

In a few cases no marked differences were observed before and after operation. Most animals, however, showed a definitely increased glucose tolerance. From experiments carried out in metabolism cages this was shown not to be due to a lowered renal threshold for glucose with attendant glycosuria. The oral administration of 5 g. glucose in 20 c.c. water is usually followed by a moderate hyperglycæmia, and at the end of 2½–3 hours the blood sugar is still slightly above the fasting level. In most hypophysectomized animals the hyperglycæmia was distinctly decreased, and in some instances the blood sugar at the end of 2½–3 hours was definitely below the fasting level. Practically the same remarks apply to the hyperglycæmic response produced by 0·2 mg. adrenaline.

Thus after a preliminary small rise the blood sugar tended to fall below the fasting value. The following results, all obtained on the same rabbit R 3, illustrate these statements:

Rabbit R 3. Given 5 g. glucose by mouth.

Before hypophysectomy (24 hr. fasting): Initial blood sugar 93 mg. p.c. Blood sugar at half-hourly intervals after glucose—180, 220, 230, 220, 190 mg. p.c.

After hypophysectomy (12 hr. fasting): Initial blood sugar 97. After glucose—92, 108, 104, 111, 104 mg. p.c.

Rabbit R 3. 0·2 mg. adrenaline subcutaneously.

Before hypophysectomy (24 hr. fasting): Initial blood sugar 115 mg. p.c. Blood sugar at half-hourly intervals after adrenaline—130, 155, 130, 115, 105 mg. p.c.

After hypophysectomy (12 hr. fasting): Initial blood sugar 96. After adrenaline—113, 108, 99, 90, 72 mg. p.c.

The response of this rabbit to insulin has already been discussed.

The absence of a normal adrenaline response cannot be attributed to a depleted store of liver glycogen, since it is also observed after a preliminary glucose feeding, just as is the abnormal insulin response.

The effect of insulin on liver glycogen storage in young hypophysectomized rabbits.

The young rabbit is unique in that it is the only animal which shows a regular and definite deposition of liver glycogen after small doses of insulin. Investigations of this phenomenon by one of us [Corkill] led to the suggestion that it was not due to an uncomplicated effect of insulin, but in all probability involved the action of adrenaline, and possibly other hormones, secreted in response to the insulin hypoglycæmia. More recent investigations, shortly to be published, in which the effect of ergotoxine has been tested, have raised a doubt as to the significance attributed to adrenaline. See also Goldblatt [1933].

In view of the possible participation of hormones from the pituitary body, it seemed of interest to study this action of insulin in young hypophysectomized rabbits. As already mentioned, operation in young rabbits is liable to be attended by a high operative mortality, so that it was impossible to obtain a large series of animals. Apart from operative trauma, our greatest loss was due to severe spontaneous hypoglycæmic symptoms, which were apt to appear within 48 hours of the operation. We managed, however, to obtain several animals which, 5 days after the operation, appeared to be fully recovered, and showed no obvious abnormality.

In Table II the values obtained on two separate litters are combined. The general plan of the experiment and chemical estimations followed that already described in a previous paper [Corkill, 1930].

TABLE II.

	Glycogen p.c.	
	Liver	Muscle
1. Normal control	0·30	0·34
2. Normal control	0·48	0·26
3. Normal, injected with 1 unit insulin, convulsed 3½ hr. later	2·55	0·24
4. Hypophysectomized control	0·16	0·15
5. Hypophysectomized, injected with 0·2 unit insulin, convulsed 1½ hr. later	0·45	0·13

In this single experiment the hypophysectomized animal showed a greatly increased sensitivity to insulin. Even though the dose was greatly reduced, so that hypoglycæmic symptoms did not occur until 90 min. later, this animal did not show any definite deposition of liver glycogen, the values obtained being clearly within normal limits.

DISCUSSION.

If we accept Houssay's main findings, and these appear to be based on careful experimental work, we must assume that the anterior lobe of the pituitary body influences carbohydrate metabolism in a sense directly opposed to that of the internal pancreatic secretion; for hyperfunction of the anterior lobe, or repeated injection of its extract, is followed by symptoms characteristic of deficient pancreatic function, namely diabetes; while, on the other hand, ablation of the anterior lobe produces a condition in many ways suggestive of excessive pancreatic function, and we might well expect that, under such conditions, the additional effect of quite a small injection of insulin would lead to the development of a severe hypoglycæmia. But the characteristic hypersensitiveness to insulin of hypophysectomized animals, with which we are here particularly concerned, cannot be due merely to an excessive secretion of insulin by the pancreas, since this would not explain why, in the absence of the pancreas, removal of the pituitary body abolishes the specific symptoms of insulin deficiency.

For a clear appreciation of the problem, it is important to remember that the course of the blood sugar after administration of insulin is determined jointly by the effect of the insulin, and by the compensatory response of the organism, which tends to restore the lowered blood sugar to the initial level. Thus there is good reason to believe [Cannon, *et al.* 1924] that when the blood sugar has fallen to a certain critical level, usually about 75 mg. p.c., a compensatory secretion of adrenaline by the suprarenals takes place, which in turn causes a discharge of glycogen from the liver and a consequent replenishment of the lowered blood

sugar. One of the commonest causes of hypersensitivity to insulin is a derangement of this compensatory mechanism, and this would seem to be effective in the present case, in view of the observed difficulty with which the animals recover from the hypoglycæmic effects of insulin.

Obviously if the glycogen reserves in the liver are depleted, the blood sugar will fail to rise. In the present case, however, such an explanation is inadmissible, for the hypersensitive rabbits with which we worked did not show evidence of glycogen depletion until the final stages of spontaneous hypoglycæmia. Even when we had ensured a plentiful supply of liver glycogen by previous administration of glucose, the hypersensitivity to insulin was still in evidence.

We must therefore look elsewhere for the breakdown in the compensatory mechanism. It is well known that removal of the pituitary body leads to a marked decrease in size of the suprarenal glands, due mainly to a degenerative shrinkage of the cortical tissue. It seemed possible, however, that the medulla might also be affected, and that the cause of the breakdown might lie in the presence in the medulla of insufficient adrenaline for the purposes of glycogen mobilization. Our comparisons of the adrenaline content of the suprarenals of normal and hypophysectomized rabbits do not, however, support this view; nor have we any reason to suspect a defective liberation of adrenaline from the glands, in response to the hypoglycæmic stimulus.

Further, whatever its cause, if lack of "available" adrenaline were responsible for the impaired recovery of hypophysectomized animals, we should expect a rapid recovery to occur when the requisite adrenaline was supplied from without. This, however, was not the case. It appears, then, that the failure of the hypophysectomized rabbit to recover from insulin hypoglycæmia is not due to the lack either of liver glycogen or of adrenaline to mobilize it, for it is still observed even when both glycogen and adrenaline are available in adequate amount. We must therefore suppose that the liver glycogen has become abnormally resistant to the mobilizing action of adrenaline, and this supposition is borne out by the impaired ability, and sometimes complete failure, of adrenaline to produce hyperglycæmia in hypophysectomized animals.

This picture of increased response to insulin and diminished response to adrenaline somewhat resembles that observed by Burn and Marks [1925] in rabbits deprived of the thyroid gland, and it may well be due in part to the degeneration of the thyroid gland, known to take place after removal of the pituitary body. The loss of resistance to insulin intoxication in the absence of the pituitary gland, however, is far more

severe than anything observed in thyroidectomized animals, and there is probably some further endocrine deficiency which aggravates the condition of the hypophysectomized animal. In view of the extreme atrophic changes in the adrenal cortex, we are tempted to suspect that cortical deficiency may play an important part. Although Britton and Silvette [1932] have published some suggestive observations, the rôle of the adrenal cortex in carbohydrate metabolism is still obscure. We are carrying on concurrently an investigation on adrenalectomized animals, which we hope to link up with the work reported here.

Summary and conclusions.

1. Rabbits from which the pituitary gland has been removed became abnormally sensitive to the hypoglycæmic action of insulin, and may even develop a spontaneous hypoglycæmia, especially when deprived of food for several hours.

2. In animals which exhibit spontaneous hypoglycæmia the glycogen reserves are found to be depleted, and this lack of available carbohydrate may be a contributory cause to the fall in blood sugar.

3. Lack of carbohydrate is not responsible for the increased response to insulin, for the latter is observed in animals which have ample reserves of liver glycogen.

4. The increased insulin response is characterized by delay in the return of the blood sugar to the normal level. The hypoglycæmic symptoms are usually severe and are relieved only with great difficulty. Injections of adrenaline or of vaso-pressin, which will usually relieve insulin hypoglycæmia in the normal animal, have little or no effect in the animal deprived of its pituitary body.

5. Animals which are abnormally sensitive to insulin usually exhibit also a diminished response to adrenaline, and an increased sugar tolerance. It is suggested that an abnormal resistance of the glycogen reserves to the mobilizing action of adrenaline is a factor in the increased sensitiveness to insulin.

6. The possibility that this stabilization of liver glycogen is consequent upon the thyroid degeneration observed after removal of the pituitary body is being investigated.

7. Young rabbits, which normally deposit liver glycogen as a result of insulin injection, fail to do so when deprived of the pituitary gland.

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A COMPARISON OF FŒTAL AND MATERNAL HÆMOGLOBINS IN THE GOAT.

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(*Received September* 19, 1933.)

Recent investigations have shown that the oxygen dissociation curves of fœtal and maternal bloods are not identical. Comparisons on the blood of goats have been published by Huggett [1927] and on human blood by Haselhorst and Stromberger [1931]. The affinity of hæmoglobin for oxygen depends upon the concentrations of hydrogen ions and other electrolytes, and in order to determine whether the differences in the dissociation curves are due to differences in the hæmoglobins, experiments have been made in this work on purified solutions of maternal and fœtal hæmoglobins, in which the concentrations of hydrogen ions and salts have been defined by the dialysis of the protein solutions against relatively large volumes of a standard buffer solution. In addition, measurements of osmotic pressure and of the membrane potential have been made in order to determine whether there is any difference between the molecular weights or the ionization of maternal and fœtal hæmoglobins[1].

Preparation of material. Samples of the blood of maternal and fœtal goats were used for the preparation of hæmoglobin. The corpuscles were washed three times with salt solution. The stromata were removed by the use of purified ether and sodium chloride, as described by Adair [1925]. The preparations were placed in collodion membranes and dialysed for 1 day with distilled water and then for periods from 4 to 10 days with the Sörensen phosphate buffer mixture containing 1/30 mol. of potassium dihydrogen phosphate and 1/30 mol. of disodium hydrogen phosphate per litre of solution. The *p*H value of this mixture is 6·81 at 20° and about 6·78 at 38° C. For the purposes of this work it seemed desirable to use the solution at *p*H 6·8 rather than at the physiological 7·4, or more alkaline reactions. In the first place, higher oxygen tensions are used for the

[1] Dilute solutions of hæmoglobin from the same series of goats were studied simultaneously by Prof. F. G. Hall using spectroscopic methods. The results were similar to those found in this paper and will be published shortly.

dissociation curve at 6·8, and the oxygen pressure at half saturation can be determined with greater accuracy from the gas analyses. A second and more important advantage is that the *p*H values of different hæmoglobin preparations are less variable after dialysis with solutions at *p*H 6·8 than at 7·4, because the phosphates have a higher buffer value at 6·8 and are less affected by traces of carbon dioxide. The effects of variations in the *p*H on the oxygen dissociation curve are smaller at *p*H 6·8 than at 7·4. Even if the *p*H values are identical at 0°, there may be variations at 38°, because the effect of temperature on the *p*H may be greater in the solutions with higher concentrations of hæmoglobin. The temperature correction is less important at *p*H 6·8 than at 7·4.

There is one disadvantage in working at *p*H 6·8; oxyhæmoglobin changes into methæmoglobin more rapidly at *p*H 6·8 than in more alkaline solutions. Estimates of the methæmoglobin formation were made by the following procedure. The refractive indices of the protein solution R' and of the dialysate R'' were measured and the protein concentration C was calculated by the formula $R' - R'' = 0{\cdot}001945\,C$ as described by Stoddard and Adair [1923]. The theoretical oxygen capacity was calculated on the provisional assumption that 1 g. of the goat's hæmoglobin is equivalent to 1·34 c.c. of oxygen, the value accepted for the hæmoglobin of the horse. The oxygen capacities were then determined and the results are recorded in Table I. Provisional estimates of

TABLE I.

	Date of pregnancy, weeks and days	Date dialysis started	Date dialysis ended	Date dissociation curve measured	Oxygen pressure in mm. at half saturation	n	Oxygen capacity	Oxygen p.c. of theoretical
Normal	—	March 25	March 31	April 7	47	2·12	14·01	92·3
Maternal	12	April 19	April 26	April 26	43	2·04 ± 0·2*	12·7	85·3
Maternal	13, 2	April 25	May 3	May 27	36	2·2 ± 0·2*	8·59	90·5
Fœtal	13, 2	April 24	May 2	May 2	31	1·7	6·76	82·8
Maternal	15, 1	May 5	May 10	May 19	39	2·25 ± 0·15*	13·36	84·7
Fœtal	15, 1	May 5	May 10	May 15	31·5	2·17	8·98	85·2
Maternal	16	June 1	June 8	July 22	42	2·35	6·87	74·7
Fœtal	16	June 1	June 8	July 13	24·5	2·17	7·18	—
Maternal	18	May 23	June 2	June 13	38	2·0	8·50	86·6
Fœtal	18	May 22	June 2	June 7	22·5	—	4·60	72·8
Maternal	19, 6	June 1	June 8	July 18	34	2·27	14·1	71·5
Fœtal	19, 6	June 1	June 8	July 17	26	2·5 ± 0·3*	8·15	73·6

* Probable error in the determination of n.

the percentage of the total hæmoglobin in the active form are recorded in Table I, column 9. These figures may require multiplication by a constant factor when goats' hæmoglobin has been subjected to further

analysis, but from the results of colorimetric determinations of total hæmoglobin it appears unlikely that the factor will exceed 1·05. It appears therefore that some of the preparations contain over 20 p.c. of methæmoglobin.

It is by no means certain that the methæmoglobin formation is wholly due to the *p*H of the buffer mixture. In a programme of work on blood as well as on hæmoglobin, the blood must be dealt with first in order to minimize the risk of changes in *p*H and it may be necessary to defer work on the hæmoglobin. There is little doubt that higher percentages of oxyhæmoglobin could be obtained if the work could be finished in a shorter time.

Methæmoglobin interferes with certain methods for the study of the dissociation curve. The experimental results described below indicate that there is little difference between the dissociation curves of solutions containing varying amounts of methæmoglobin. Since the amounts of methæmoglobin formed in maternal and fœtal hæmoglobins, dialysed at the same time are the same, there is no reason to suppose that methæmoglobin formation vitiates the comparison of maternal and fœtal hæmoglobins.

Oxygen dissociation curves.

For the measurement of the oxygen dissociation curves, the hæmoglobin solutions were equilibrated at 37° C. in the new Barcroft saturator[1] with an enclosed double bulb pipette. Duplicate Haldane analyses were made on the gas phase, and the percentage oxygenation of the solutions was determined by the van Slyke manometric method.

The fœtal and maternal hæmoglobin solutions were obtained from goats in the 12th–20th week of gestation. The total period of pregnancy in the goat is 21 weeks. A non-pregnant goat was used as a control. The characteristics of the hæmoglobin solutions used are shown in Table I.

The oxygen dissociation curves of a number of preparations are shown in Fig. 1, and experimental data for the oxygen dissociation curves of additional preparations are recorded in Table II. The curve numbered 1 is a composite curve of all the maternal hæmoglobins. The range of variation covered by the points is comparatively small, and it appears that the oxygen dissociation curves of all the maternal hæmoglobins are nearly identical. The pair of curves, numbered 2 and 3, represent the fœtal and maternal hæmoglobin obtained at the 16th week of pregnancy. The fœtal and maternal hæmoglobins obtained at the 18th week of pregnancy are represented by curves 4 and 5, and those obtained at the 20th

[1] A description of this apparatus will be published later.

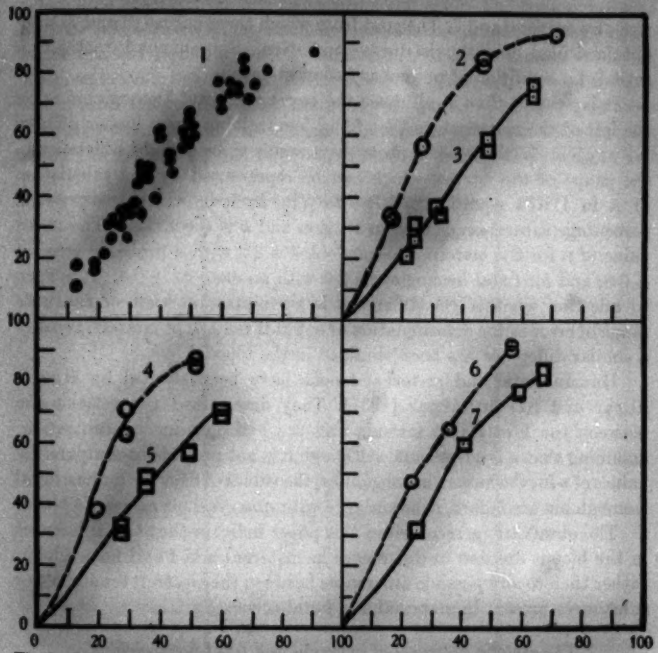

Fig. 1. Oxygen dissociation curves of hæmoglobin of goats. Ordinates: percentage oxygenation. Abscissæ: oxygen pressures, mm. of Hg. Circles: fœtal. Squares: maternal. Interpretation in text.

TABLE II. Percentage oxygenation and oxygen pressures in mm. of Hg.

Normal	Pressure in mm.	33·6	38·7	44·2	69·0	—	—
	Saturation p.c.	34·0	39·0	47·0	70·8	—	—
Maternal,	Pressure in mm.	29·0	43·5	51·0	90·5	—	—
12th week	Saturation p.c.	26·4	51·3	59·0	87·0	—	—
		35·6	53·0	62·0	—	—	—
Fœtal,	Pressure in mm.	26·4	13·2	50·0	80·7	—	—
13th week	Saturation p.c.	36·6	19·6	79·1	74·4	—	—
		45·8	21·4	66·0	85·9	—	—
Maternal,	Pressure in mm.	12·8	27·9	33·7	49·5	71·0	—
13th week	Saturation p.c.	10·10	32·9	47·2	64·5	75·5	—
		17·85	34·5	49·5	67·0	76·5	—
Fœtal,	Pressure in mm.	14·3	26·3	29·0	55·5	—	—
15th week	Saturation p.c.	13·0	37·4	42·2	78·0	—	—
		16·3	38·0	44·5	73·0	—	—
Maternal,	Pressure in mm.	19·3	30·3	32·8	65·4	48·0	75·2
15th week	Saturation p.c.	15·9	34·3	44·3	73·5	60·7	80·5
		17·5	35·4	44·6	75·0	—	—

week by curves 6 and 7. The goat from which these curves (6 and 7) were obtained died of asphyxia during operation. Subsequent investigation revealed a condition of pulmonary congestion.

It is evident that in all cases the curve for fœtal hæmoglobin is on the left of the maternal, so that fœtal hæmoglobin has a higher affinity for oxygen. In the second place there seems to be a slight difference in the shape of the curves, which can be represented by the calculation of n in Hill's equation, $y/100 = kx^n/(1 + kx^n)$, in which y represents percentage saturation, x oxygen tension and k is a constant. The mean value of n for the maternal hæmoglobins is 2·2 with a probable error of $\pm$ 0·3, and for fœtal hæmoglobins 2·0 with an error of $\pm$ 0·4. It is open to question whether the difference is significant, in view of the wide range of error in the determination of n, but it may be of interest, because a similar difference has been obtained in the bloods[1].

Human fœtal and maternal bloods have been studied by Haselhorst and Stromberger [1931]. They determined the relationship between the k of Hill's formula and the hydrogen-ion concentration, assuming that n is a constant. Although it is not possible to compare the values of n for the human hæmoglobins, the values of k for the human fœtal hæmoglobin are higher, in accordance with observations on goat's blood.

The observations recorded in this paper indicate that the differences in the bloods are due to differences in maternal and fœtal hæmoglobin rather than to any possible differences between the electrolytes and other substances present in maternal and fœtal corpuscles.

The osmotic pressures of maternal and fœtal hæmoglobins.

In view of the difference between the dissociation curves of maternal and fœtal hæmoglobins, a number of measurements of the osmotic pressure of the proteins were made, using the same phosphate buffer mixture. Osmometers with collodion membranes were used as described by Adair [1925]. The protein concentrations were determined by the refractometric method. The observations are recorded in Fig. 2. The continuous curve in Fig. 2 represents the osmotic pressure of the hæmoglobin of the sheep [Adair, 1928]. The circles represent observations on the fœtal hæmoglobin of the goat, and the squares on the maternal hæmoglobin. It appears that both of these forms have osmotic pressures in fairly close agreement with the data for sheep's hæmoglobin, which has a molecular weight of 68,000. Most of the points for fœtal hæmoglobin lie above the curve, but it is not unlikely that the relatively small

[1] Observations on blood made in this laboratory.

differences observed are due to experimental error. The results in Fig. 2 may be subject to a slight error, because the calculations of the protein concentration depend upon the assumption that the refraction value of a 1 p.c. solution is 0·001945, as in the case of the hæmoglobin of the sheep.

In addition to these measurements of osmotic pressure, a number of measurements of the membrane potential have been made, and from these the ratio E/C_v has been calculated as described by Adair and

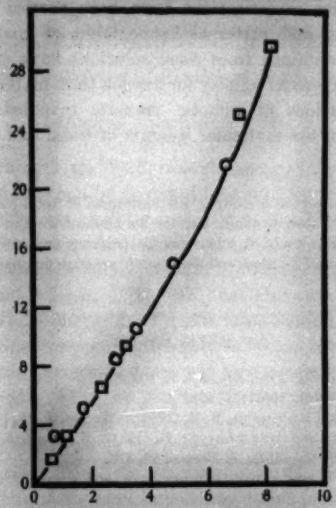

Fig. 2. Ordinates: osmotic pressure in mm. of mercury at 0° C. Abscissæ: concentrations in grams of protein per 100 c.c. of solution. The smooth curve is copied from the curve for sheep's hæmoglobin given by Adair [1928]. The circles are fœtal hæmoglobin, squares maternal hæmoglobin.

Robinson [1930]. E represents the membrane potential in millivolts and C_v the corrected concentration of the protein in grams per 100 c.c. of solvent. In the case of the maternal hæmoglobin the following values of E/C_v were obtained:

0·057, 0·055, 0·058, 0·075, 0·057.

In the case of fœtal hæmoglobin the ratios obtained were:

0·099, 0·077, 0·105, 0·091.

In all cases the protein solutions were negative, the protein acting as an anion in the buffer mixture at *p*H 6·9 at 0° C. It appears that the fœtal hæmoglobin has more negative charge than the maternal hæmoglobin at the same *p*H, but the potentials observed were so small that the difference might possibly be due to experimental error.

Summary.

1. Comparative investigations have been carried out on purified solutions of fœtal and maternal hæmoglobin of goats. Oxygen dissociation curves obtained from these solutions have shown that fœtal hæmoglobin has a greater affinity for oxygen than maternal hæmoglobin.

2. Determinations of osmotic pressure revealed no appreciable difference between the molecular weights of fœtal and maternal hæmoglobins.

This work forms a part of a study of the conditions of fœtal respiration planned by Prof. Barcroft, and I wish to thank him for his kind advice and help. I also wish to express my gratitude to Mr G. S. Adair for his generous and invaluable assistance in supervising the dialysis of solutions and the osmotic pressure determinations.

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612.741.4

THE EFFECT OF MUSCLE LENGTH ON THE ENERGY FOR MAINTENANCE OF TENSION.

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(*Received September* 5, 1933).

It has been shown by Hill [1925] that the energy liberated by a muscle during a short tetanus can be analysed into heat for the development of tension and heat for the maintenance of tension. Thus the initial heat produced in a 0·3 sec. tetanus is greater than that produced in a 0·1 sec. tetanus by an amount which represents the energy needed to maintain tension for the last 0·2 sec. Hill [1925] has also shown that the initial heat in a twitch, or the energy needed to develop tension, is at a maximum at a muscle length corresponding roughly to 90 p.c. of the resting length of the muscle, while the tension developed by the muscle is at a maximum at the resting length. It is the purpose of this paper to show that the energy to maintain tension is affected by changes in the length of the muscle in the same manner as the energy to develop tension.

The behaviour of this "maintenance energy" at different muscle lengths has a certain theoretical importance which prompted the present investigation. It was shown by Fenn [1923] that an extremely small shortening (3 p.c.) of a muscle in an otherwise isometric contraction may lead to a considerable (18 p.c.) increase in heat production, and, further, that the internal work in a gastrocnemius muscle due to the inevitable shortening of the fibres, even when the muscle as a whole is rigidly isometric, causes enough extra heat to account for the dissimilarity in the heat production of the sartorius and gastrocnemius muscles in isotonic contractions. Even in a sartorius muscle, however, the "isometric" contractions involve some internal work which no one has yet even attempted to evaluate and which may be of great importance, particularly when isometric heats at different lengths are compared. The internal work certainly would vary with the length of the muscle and

might account in part, or even *in toto*, for the variations of heat with length of muscle. If this were the case, then presumably the energy for maintenance of tension would not vary with the length of the muscle, as does the energy for the development of tension, for it is liberated after the internal work is over.

Method.

Single sartorius muscles from *Rana pipiens* were used as material. The thermopile was one designed by Bozler [1930] for use with smooth muscle and had a single row of hot junctions. The upper, tibial end of the muscle was fastened to the isometric lever by a fine drill rod to avoid stretching. The isometric lever was of the torsion wire type and was equipped with an extra writing point fixed to the frame to record zero tension. The increase in length of the muscle was measured from the amount by which the lever was raised. The resting extended length of the muscle in the frog was measured in most experiments and found to correspond fairly well with the length for maximum development of tension. The thermopile chamber was kept immersed in a "thermos" jug at room temperature. The thermopile was equipped with three electrodes, one near each edge of the thermopile and one near the upper end of the muscle. The middle and bottom electrodes were used for calibration and the top and bottom for stimulation. A.C. house current (60 cycles per sec.) cut down by a transformer and potentiometer was used for stimulation, duration being measured by a rotary contact breaker. Calibration was carried out mostly by Bozler's [1931] condenser method as applied by A. V. Hill [1931]. Heat was calculated for that part of the muscle lying between the middle and botton electrodes, no correction being made for the muscle beyond the electrodes. The length of muscle thus considered was 2·5 cm. long.

The stimulus was adjusted to be just maximal as judged by the tension developed. Observations began at the shortest length, the heat for 0·1 and 0·3 sec. tetanus (or 0·2 and 0·4 sec.) being measured. Then the muscle was stretched in successive steps of 3 mm. each and stimulated for 0·1 and 0·3 sec. at each step until the resting tension began to increase considerably and the total tension development began to decrease. Finally the same series was repeated in the reverse order. If a good return series was not obtained the experiment was discarded. The "up" and "back" series were averaged together. Nine successful experiments were performed giving twenty series.

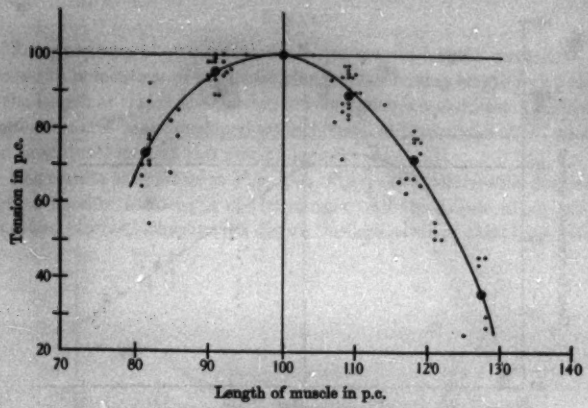

Fig. 1. Tension in p.c. of the maximum at different muscle lengths.

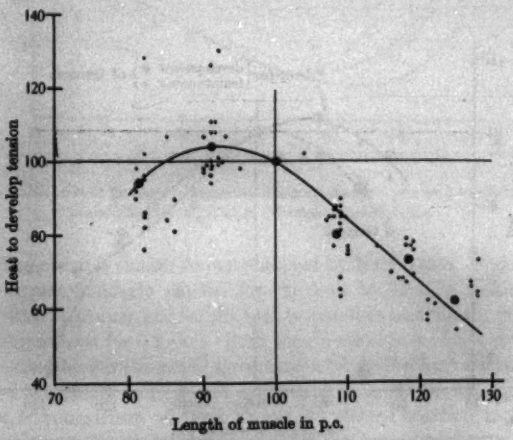

Fig. 2. Heat for the development of tension at different muscle lengths.

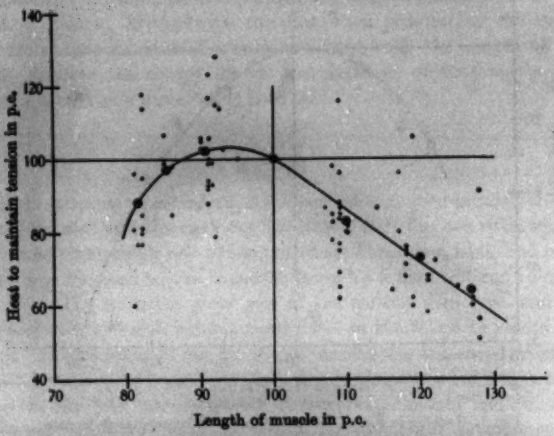

Fig. 3. Heat for the maintenance of tension at different muscle lengths.

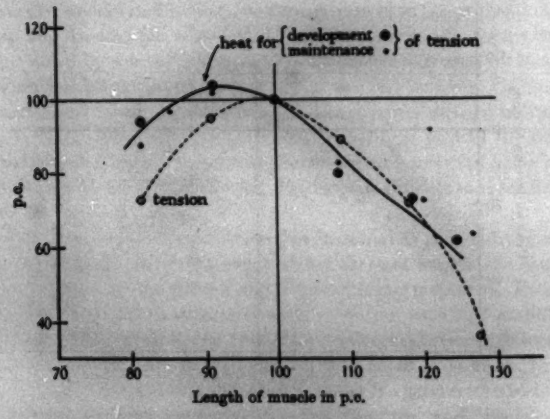

Fig. 4. Average points from Fig. 1–3 plotted together for comparison.

Results.

For purposes of presentation all the muscle lengths have been expressed in percentage of the resting length, the resting length being taken as the length at which the tension development is a maximum. Likewise the tension and heat developed are expressed in percentage of the tension and heat developed at this resting length.

The results are plotted in Figs. 1–4. Fig. 1 shows the tension developed plotted against the length of the muscle. All the points of all twenty series are plotted. The curve is drawn through average points marked by

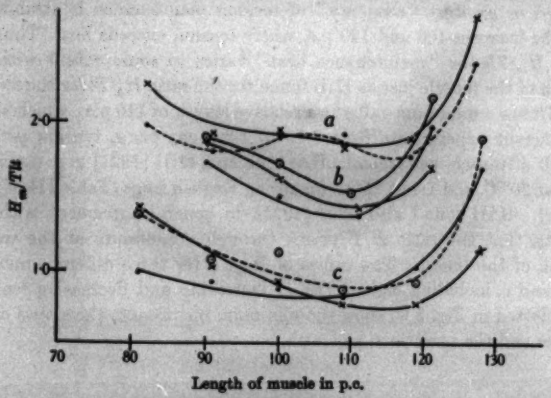

Fig. 5. Ratio of heat for the maintenance of tension (per cm. per sec.) to the tension maintained (or H_m/Tt) at different muscle lengths.

circles. The curve is similar to one obtained by Hill [1925]. Figs. 2 and 3 show curves similarly plotted for the heat to develop tension, H_d (0·1 or 0·2 sec. tetanus), and for the heat to maintain tension, H_m (heat for 0·4 sec. minus heat for 0·2 sec.). Both show a maximum at about 90 p.c. of the resting length (in exact agreement with Hill's heat curves), the scatter of the points being somewhat greater than for the tension. For purposes of comparison all three curves are plotted together in Fig. 4. The heat curves for maintenance and for development of tension are identical within limits of error of the method. This shows that the conditions, whatever they may be, which cause the heat of a twitch (or the

heat to develop tension) to vary with muscle length must obtain equally during the maintenance of tension. The heat associated with the varying internal work at different lengths is therefore presumably not involved unless there is some internal work due to alternate contraction and relaxation of the ultimate contractile units within the individual muscle fibrils during the maintenance of tension in a tetanus[1].

The heat necessary to maintain 1 g. of tension in 1 cm. of muscle for 1 sec., H_m/Tlt, has been calculated for all these experiments. Inspection of Fig. 4 shows that H_m exceeds T (both expressed in percentage) at very short and very long muscle lengths indicating that more heat is needed at these points for the maintenance of the same tension. Least heat is needed or greatest "economy" of tension maintenance is attained at lengths between 100 and 120 p.c. where tension exceeds heat. Thus the ratio H_m/Tlt for "maintenance heat" varies to some extent with the length of the muscle just as Hill found for the ratio H_d/Tl for the twitch. H_m/Tlt has a minimum value at a relative length of 110 p.c., which varies in different experiments from 0·75 to 1·9 g. cm. per g. tension per cm. length of muscle per second. Hartree and Hill [1921] give figures of 0·92 at 20° C. and 1·4 at 25° C. for spring English frogs (Table III of their paper). Hill found also later [1925], in general agreement with our results, that the ratio H/T passes through a minimum at the resting length of the muscle. The values of H_m/Tlt for three different muscles, *a*, *b*, and *c*, including eight series of increasing and decreasing lengths, are plotted in Fig. 5 to show the minimum mentioned, the slopes of the curves and the variations met with.

Summary.

The energy necessary to maintain 1 g. of tension in 1 cm. length of frog's sartorius muscle for 1 sec. has been measured at different muscle lengths. The energy for the maintenance of tension is found to vary with changes in muscle length in exactly the same way as the energy for the development of tension. This would indicate that variations in internal shortening of the fibres at different lengths do not account for the variations in heat observed, since such shortenings are presumably all complete when the maintenance heat is being measured.

[1] It may be necessary to consider this apparently remote possibility seriously, for an estimate of the amount of internal work at different muscle lengths suggests that it is large enough to explain the variation of heat with length. This work, however, is not yet complete.

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THE PHOSPHORUS DISTRIBUTION IN RESTING FLY MUSCLE.

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(*Received September* 21, 1933.)

Introduction.

Representative members of nearly all the invertebrate phyla have now been examined for their phosphagen content [Eggleton and Eggleton, 1928; Meyerhof, 1928; Needham, Needham, Baldwin and Yudkin, 1932]. It has been established that creatinephosphoric acid is characteristic of the Vertebrata, while argininephosphoric acid is restricted to the Invertebrata. The two compounds have only been found to co-exist in the Echinoids and in the Enteropneusts. This is of especial interest, since the latter (Hemichordates) form a morphological link between the vertebrate and the echinoderm phyla. Another protochordate group, the Ascidians (Urochordates), have recently been examined by Kreps [1933], who finds creatinephosphoric acid and no argininephosphoric acid; they thus resemble *Amphioxus* (Cephalochordates).

It must be remembered that only in the case of one species (the crayfish, *Astacus fluviatilis*) has arginine phosphoric acid actually been prepared from invertebrate muscle and analysed [Meyerhof and Lohmann, 1928]. In most cases the evidence for its presence consists in the demonstration of a phosphorus compound, stable in strong acid in presence of molybdate, but hydrolysing in 18 hours at 28° C. in *N*/20 acid in absence of molybdate. In the cases of *Pecten*, *Holothuria* and *Sipunculus*, Meyerhof has supported this evidence by showing that the base of the phosphagen (after hydrolysis) is attacked by arginase with production of urea. It is thus evident that much more work is needed on invertebrate phosphagen. Baldwin [1933] has brought forward evidence suggesting that the cephalopod phosphagen has different properties from argininephosphoric acid, and Arnold and Luck [1933]

have pointed out the possibility that a hitherto unknown phosphagen is contained in the polychaete worms.

Curiously enough, one large and important class of Invertebrata had, until very recent times, remained untouched—namely, the Insecta. The wing muscles of the flying insects are of special interest, as their rate of contraction and relaxation is said to be quite unparalleled in the animal kingdom. According to Marey [1873] in the house-fly the wings make 330 complete vibrations per second. We therefore took up the study of insect phosphagen, choosing *Calliphora* and *Lucilia* as good representatives of the flying insects in which rapidly acting musculature has reached its highest development. Besides this object, we also had the purpose of investigating the distribution of the whole acid soluble phosphorus and of following the changes in the fractions during fatigue and rigor. This had been done in a systematic manner only for the frog and the rat. The present paper describes the results on resting muscle; the work on stimulated muscle and on muscle in rigor is in progress. When this work was nearly finished, a paper by Schütze [1932] appeared; he had examined various insect muscles (*Locusta*, *Dytiscus*, *Hydrophilus*, *Lucanus*, *Apis*, *Aeschna*), and reported the presence of argininephosphoric acid, relying, however, only on the evidence from rate of hydrolysis.

The flies used in our work were raised from larvæ which were placed in suitable cages in a warm, well-lighted room. Pupation, hatching and breeding took place in due course; the adults were fed on sugar, water and liver. The insects were collected for the experiments in tall narrow beakers; this was easily accomplished by darkening the whole cage, applying the beaker to an opening, and illuminating the end of the beaker with a bright lamp. When enough insects had entered the beaker, the latter was covered by means of a Petri dish and entirely surrounded for some minutes in a freezing mixture. This treatment rapidly immobilized the insects, which were then tipped out into a small, cooled beaker to await dissection. We have assured ourselves that this treatment need not injure the flies; for if a few of the cooled insects were allowed to warm up, after not too long a time at the low temperature, rapid and apparently complete recovery followed. It was not feasible to dissect out the thoracic muscles individually, but as the thorax consists almost entirely of muscle, surrounded by a thin chitinous covering, this whole region was taken for analysis.

The amount of skeletal tissue in the thoraces was estimated by Buxton's method [1932]. The thoraces were dried at 100° C., finely powdered, then heated in a boiling water bath, under a reflux, for 5 hours

with 1 p.c. KOH. The contents of the flask were filtered through a weighed Gooch crucible, which, after washing, was dried and reweighed. From 3·3 g. of fresh tissue (150 flies) 0·119 g. of dry residue was obtained; so that 3·6 p.c. of the weight of fresh thorax is to be regarded as consisting of skeletal tissue. It was not possible to estimate the weight of the thoracic viscera, but it is quite clear from the data given by Lowne [1890–2] (see especially Plate XI) that it is very small in comparison with the muscle weight, certainly not more than about 5 p.c. of the fresh thorax weight. All the results are expressed in mg. per 100 g. of fresh thorax.

When the insects were completely motionless, they were placed, a few at a time, on a cooled glass plate, and the head, abdomen, wings and legs were dissected off from each. The thoraces were extracted at 0° C. by grinding thoroughly with ice-cold 5 p.c. trichloroacetic acid (usually about 5 c.c. per g. of tissue) and quartz sand. The extract was then filtered under pressure into a cold centrifuge tube and the clear, sparkling, slightly yellow filtrate was used for the experiments described below.

All the phosphate estimations were done by Fiske and Subbarow's method [1929], using a Klett colorimeter. The separation of the phosphate fractions into those with insoluble calcium and barium salts (free phosphate and adenylpyrophosphate) and those with soluble calcium and barium salts (phosphagen and hexosemonophosphate) was performed sometimes by means of Fiske and Subbarow's $CaCl_2$—$Ca(OH)_2$ reagent, sometimes by means of finely powdered baryta and baryta water [Eggleton and Eggleton, 1929–30; Cori and Cori, 1931–2*a*].

Phosphagen.

Evidence for the absence of creatinephosphoric acid from the cold extract and for the presence of a substance with a rate of hydrolysis resembling that of argininephosphoric acid was obtained in the following way. The preformed inorganic phosphate in a portion of the neutralized extract was precipitated by the Fiske and Subbarow calcium reagent, the precipitate was dissolved in acid, and the phosphate estimated. A second portion of the extract was allowed to stand 15 min. with the molybdate reagent before the aminonaphtholsulphonic acid was added. This treatment should cause complete breakdown of any creatinephosphoric acid present, but no rise in inorganic P was found. A third portion was diluted with water or partly neutralized so as to be about $N/20$ in acidity, and then incubated at 28° C. overnight. An increase in inorganic phosphate was then found, and this increase may be regarded as the argininephosphoric

acid P. In amount it varied between 10 and 20 mg. per 100 g. The preformed inorganic phosphate in the same experiments varied between 17 and 25 mg. per 100 g.

In other cases the centrifugate after removal of the insoluble calcium salts was used. Here inorganic phosphate was not initially present, but was set free during the incubation period. In order that the base combined with phosphate might be further examined, apart from any free bases present, such a centrifugate was treated with two volumes of ice-cold alcohol; a precipitate consisting of the calcium salt of the phosphagen together with the calcium salt of a hexose ester was obtained, which, after standing, was spun off, washed and then dissolved in normal acid. It was left at room temperature overnight, so that phosphagen present might break down, and then tested:

(*a*) By Weber's modification [1930] of the Sakaguchi reaction for arginine. This gave a good colour, corresponding roughly to the amount of labile phosphorus which had been found in the extract. No free base was present before incubation.

(*b*) By Walpole's diacetyl test [1911], which was negative. Controls showed that the calculated amount of creatine (from the labile phosphorus) gave a good pink colour. The calculated amount of arginine gave no colour.

(*c*) The Jaffé reaction was negative.

We next investigated, by two methods, the effect of arginase (prepared according to Edlbacher and Röthler's directions [1925]) on the base combined in the phosphagen.

(*a*) *By the Weber-Sakaguchi method.* The precipitate containing the calcium salts of the phosphagen and hexose ester was prepared from about 400 flies, as described above. It was dissolved in decinormal acid and heated in a boiling water bath for 15 min. to hydrolyse the phosphagen. After cooling and neutralizing, two volumes of alcohol were again added; this time the calcium salt of the hexose ester was precipitated, the base, being now free from phosphate, remaining in solution. The calcium precipitate was well washed, and the washings added to the centrifugate.

After removal of the alcohol, the solution was made up to 50 c.c. By the Weber-Sakaguchi reaction it was shown to contain 0·025 mg. arginine per c.c. A standard arginine solution of the same strength was made up. 5 c.c. of each, the standard arginine and the extract, were then incubated at 37° C., each having received one drop of the diluted arginase solution, and a few drops of toluene. The *p*H was about 9. It is usual to

employ a glycine buffer with arginase to control the *p*H, but this had to be omitted in these experiments, since glycine interferes with the Weber-Sakaguchi reagents. After 2 hours, the amount of base present, from the intensity of the colour with the Weber-Sakaguchi reagents, had been reduced to about one-eighth of that present before incubation; the residual coloration was the same in both tubes, and was not reduced by further incubation. This residual colour was due to the presence of proteins in the arginase solution, as was shown by control experiments. After deproteination of the test solutions by means of trichloroacetic acid, no coloration was produced.

(*b*) *By the urease method*, in which the production of urea by the action of arginase is shown by the action of urease on this urea. 123 flies (giving 3 g. of thoraces) were extracted with 24 c.c. trichloroacetic acid. The insoluble calcium salts were removed, and to the centrifugate two volumes of alcohol were added as usual. The precipitate was dissolved in a little HCl, made approximately decinormal, and incubated overnight at 37° C. to hydrolyse the phosphagen. The solution was then neutralized and made up to 10 c.c., 4·5 c.c. were treated with 2·2 c.c. glycine buffer (*p*H 9·0) and 0·3 c.c. of diluted arginase solution. The whole was incubated for 3 hours under a layer of toluene at 37° C. The solution was then boiled to destroy the enzyme and brought to *p*H 7, when a few drops of Folin's pyrophosphate buffer were added. 12 mg. of urease (finely powdered) were added, and incubation for 1 hr. at 37° C. followed. The ammonia was distilled off by Watchorn and Holmes' modification [1927] of Stanford's method [1923], and estimated, after Nesslerization, in the colorimeter. Blanks were performed on the enzyme solutions, etc., without the extract and on the extract alone. A similar experiment was carried out on an arginine solution (0·005 p.c.) in order to see what yield of ammonia might be expected with these very small quantities of arginine (Table I).

TABLE I.

	mg. NH_3-N found
4·5 c.c. extract without enzymes	0·011
Arginase, urease, etc., without extract	0·009
Reagents alone	0·002
Total blank	0·022
4·5 c.c. extract + enzymes	0·088
∴ NH_3N from arginine of extract	0·066
4 c.c. 0·005 p.c. arginine solution without enzymes	0·004
Arginase, urease, etc., without arginine solution ...	0·009
Reagents alone	0·002
Total blank	0·015
4 c.c. 0·005 p.c. arginine solution + enzymes ...	0·037
∴ NH_3N from arginine in solution	0·022

From 1 c.c. of the same extract it was found that 0·022 mg. free P was present per c.c. This had all arisen by breakdown of argininephosphoric acid, so that the presence of 0·124 mg. arginine, and the production of 0·020 mg. NH_3-N per c.c., were to be expected. 73 p.c. of this amount was actually found. 4 c.c. of the standard should yield 0·032 mg. NH_3-N; 69 p.c. of this amount was obtained. Thus both the Weber-Sakaguchi method and the arginase-urease method show that the base of the phosphagen is attacked by arginase.

Further evidence for the fact that the base in question is arginine is given by the rate of hydrolysis of the phosphagen in presence of molybdate ions. When muscle extracts were incubated, as usual in $N/10$ acid, in the presence of 0·3 p.c. ammonium molybdate (the concentration used in the experiments of Meyerhof [1928]) an unexpected difficulty was encountered in the phosphate estimations, which was traced to the presence of small amounts of silicate in the various reagents. The molybdate appeared to have the effect of greatly increasing the rate of liberation of phosphate, but it was soon observed that the amounts estimated as set free were impossibly high, being in some cases greater than the total P present. The explanation seems to be that silicate unites with molybdic acid in decinormal acid solutions to give silicomolybdate; the yellow colour consequent on this reaction is used as the basis of an estimation method for silicate. The silicomolybdate, we find, can be rapidly reduced in more strongly acid solutions by aminonaphtholsulphonic acid to give a blue compound, thereby simulating phosphoric acid. The formation of silicomolybdate does not take place in more acid solutions and consequently the appearance of spurious phosphate only occurs in the Fiske and Subbarow method if it is used on a solution which has previously been treated with molybdate in the presence of $N/10$ acid. Fiske and Subbarow, indeed, showed that the presence of silicate in a phosphate solution may retard the production of colour in their method.

Instead of using some other method of phosphate estimation, we decided to follow the effect of molybdate on hydrolysis rate by means of the arginine set free, using the Weber-Sakaguchi method (which gives no colour with argininephosphoric acid), in the manner described by Baldwin [1933].

Fifty thoraces (which weighed 0·98 g.) were extracted with 5 p.c. trichloroacetic acid. After filtering, the solution was diluted to 20 c.c., and soda was added until thymol blue, used as external indicator, showed the *p*H to be about 1·5. The solution was divided into parts: to 10 c.c., 1 c.c. 3 p.c. ammonium molybdate was added; to another 6 c.c.,

0·6 c.c. water was added. The two solutions were stoppered and incubated at 28° C. Samples of 0·5 c.c. were removed at intervals, and the free arginine was estimated. The method is not so dependable as the Fiske and Subbarow phosphate method, but the results, shown in the accompanying curves (Fig. 1), enabled us to calculate the following velocity constants:

0·3 p.c. molybdate present	$k = 0{\cdot}58 \times 10^{-3}$
Control	$k = 7{\cdot}7 \times 10^{-3}$

(k values are expressed in natural, not decadic logarithms)

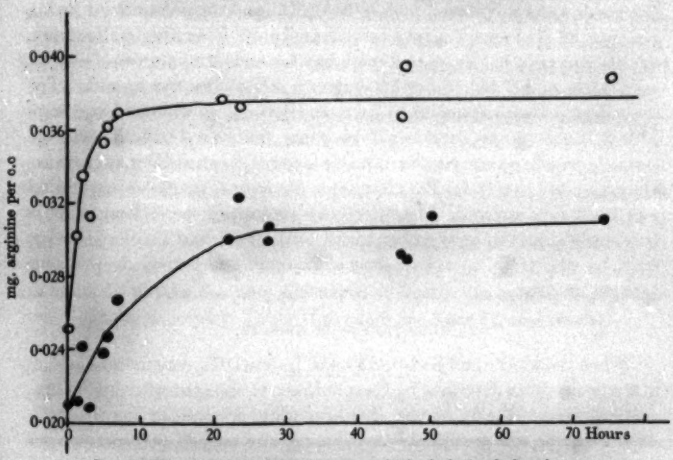

Fig. 1. The effect of molybdate ions on the hydrolysis of phosphagen.
○—○ without molybdate; ●—● with 3 p.c. ammonium molybdate.

There is thus a retardation of 14 times in the presence of the molybdate ion. Meyerhof and Lohmann [1928] and Meyerhof [1928] find that the retardation so produced in crude extracts is of the order of 15, with which our result is in good agreement; our absolute k values also agree well with those of the German workers.

All the evidence available, therefore, points to the presence of arginine-phosphoric acid in fly muscle. This could only be made certain (*a*) by the preparation and analysis of the pure compound, or (*b*) by the preparation of a crystalline derivative of the base suitable for X-ray analysis. So far, sufficient quantities have not been obtained for either of these methods.

One crystalline derivative, the flavianate, was indeed prepared, but this, owing to the large size of the flavianic acid molecule, is not suitable for distinguishing between arginine and some closely similar compound either by gravimetric or X-ray analysis. Crystalline compounds of arginine in combination with a small molecule, *e.g.* the nitrate, are far more difficult to obtain when only a few mg. of arginine are available.

To obtain the flavianate, 400 flies were used. The insoluble barium salts were removed, and the centrifugate was treated with two volumes of alcohol. After standing in ice for 2 hours, the solution was centrifuged. The residue was well mixed with $N/10$ H_2SO_4, and the precipitated $BaSO_4$ was spun off. The centrifugate was incubated at 28° C. overnight. Next day, the *p*H was adjusted again to 9, with baryta, and two volumes of alcohol were again added to remove the calcium salt of the hexose ester. The centrifugate was evaporated to 7 c.c. and 100 mg. of flavianic acid were added. The flavianate came out on standing, first as red balls. These were centrifuged off and recrystallized from boiling H_2O, when their appearance approximated closely to that of arginine flavianate, prepared in a similar way from pure arginine. Identification by means of the melting point is not possible, as the only datum given by Kossel and Gross [1924] is that, by 258–260° C., arginine monoflavianate has turned deep brown without melting.

Adenylpyrophosphate.

When the Fiske and Subbarow $CaCl_2$—$Ca(OH)_2$ reagent was added, in the proportions described by these authors, to the neutralized trichloroacetic extract of the fly muscle, a copious white precipitate was obtained. After standing this precipitate was spun off, and washed as recommended by Fiske and Subbarow. This precipitate contains all the preformed inorganic phosphate of the muscle; in the case of the frog such a precipitate contains besides the inorganic phosphate only adenylpyrophosphate [Eggleton and Eggleton, 1929–30]. The pyrophosphate is estimated by means of the increase in free phosphate after 7 min. hydrolysis at 100° C. in N HCl; in the frog the difference between the total phosphate and the free phosphate after 7 min. hydrolysis is the adenylic acid phosphorus, and amounts to half the pyrophosphate phosphorus. In the case of the rat, examined by Cori and Cori [1931–2*a*], the composition of the precipitate was found to vary according to the *p*H at which the precipitation took place. The ratio of the readily hydrolysable (pyro) phosphate to the difficultly hydrolysable was never 2 to 1 if the *p*H was higher than 7·4, but always less than 2 to 1. They showed this to be due

to the adsorption of the calcium salt of hexosemonophosphate on the precipitate, the adsorption being greater the higher the *p*H. Washing would not remove the hexosephosphate from the precipitate. It was necessary to dissolve it and reprecipitate to obtain it free from this contamination. The method adopted by the Coris was to precipitate with barium hydroxide at *p*H 8·6; this precipitate was dissolved in acid and again precipitated at 8·6. This difficulty is not encountered with the frog, as the amount of hexosemonophosphate present is negligible—2–3 mg. P/100 g. instead of about 10 as in the rat. With the fly muscle, which, as we shall see, also contains large amounts of hexose ester, we had considerable difficulty. Precipitation with the Fiske and Subbarow reagent, which had been used during the phosphagen estimations, had to be abandoned as the *p*H was too high. Even when the method of the Coris was followed, the ratio of readily hydrolysable to difficultly hydrolysable phosphate was much too low. We found, however, that by precipitating with finely ground baryta at *p*H 7·4–7·8, then dissolving and re-precipitating at this *p*H, a precipitate was obtained in which the ratio of readily hydrolysable phosphorus to difficultly hydrolysable phosphorus was exactly 2 to 1. The disadvantage of this procedure is that the inorganic phosphate is not completely precipitated at this *p*H, so that, to obtain the true preformed inorganic phosphate, a separate estimation is necessary. Table II summarizes some typical results.

TABLE II.

Exp.	*p*H	Precipitant	I	$(I+\pi)$	T	π	α	π/α
34	10	Ca (F. and S. reagent)	31·0	65·7	99·14	34·7	33·44	1·03
36	10	Ca (F. and S. reagent)	38·1	62·8	84·7	24·7	21·9	1·13
37	9	Ba	21·5	35·3	46·8	13·8	11·5	1·20
39	9	Ba	36·8	67·8	90·1	31·0	22·3	1·39
38	8	Ba	44·6	78·1	94·9	33·4	16·8	1·99
43	7·8–8	Ba	27·6	58·8	73·8	31·2	15·0	2·08
44	7·4	Ba	20·0	50·9	67·2	30·9	16·3	1·90
50	7·4	Ba	23·3	65·4	86·4	42·1	21·0	2·00

The results are expressed in mg. P/100 g. thorax.
I = inorganic, π = pyro, T = total in precipitate, $\alpha = T - (I+\pi)$.

We followed the hydrolysis of the adrenylpyrophosphate in *N* HCl at 100° C. over many hours, and obtained curves very similar to those of Lohmann [1928 *b*] (Figs. 2, 3).

In the particular experiment shown in Fig. 2 the precipitate obtained by two precipitations at *p*H 7·4–7·6 from 120 flies was dissolved in the least possible amount of *N*/10 HCl. The solution was diluted and barium

was removed by adding a concentrated sodium sulphate solution till precipitation was just complete and then centrifuging. To the centrifugate 2 c.c. of 10 *N* HCl were added and the volume was made up to 20 c.c. Samples of 1 c.c. were placed in 10 c.c. volumetric flasks, which were weighted so that they could be suspended in a rapidly boiling water bath. One minute after immersion the flasks were securely corked. They were removed at intervals, and each was placed in ice at once. The

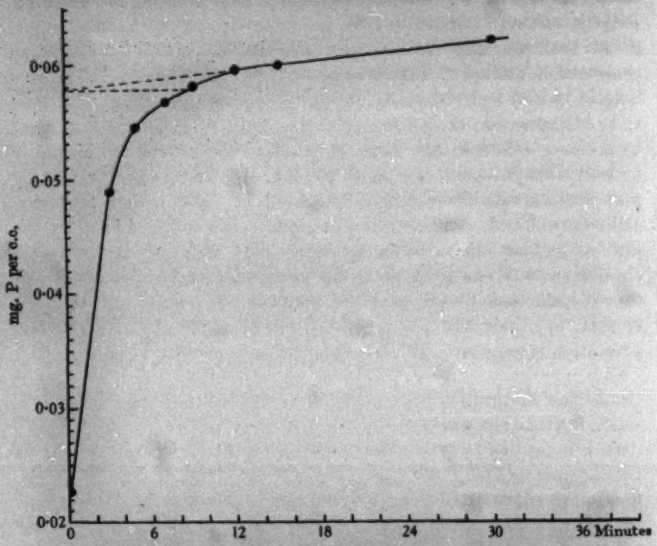

Fig. 2. Exp. 43. Hydrolysis of adenylpyrophosphate in *N* HCl at 100° C.

amount of free phosphate present in each was determined, as well as the initial phosphate and the total phosphate. For the latter the procedure used by Eggleton and Eggleton [1929–30] was followed: 1 c.c. of the extract was heated with 1 c.c. 10 *N* H_2SO_4 till white fumes appeared, then 2 drops of Merck's perhydrol were added. Gentle heating was continued until the liquid was quite colourless and until all effervescence was ended. After cooling, a little water was added, and the contents of the tube were boiled for 2 min. to decompose any remaining hydrogen peroxide, and any pyrophosphate and persulphate formed during the incineration. In

performing the phosphate estimation, it was necessary to allow for the H_2SO_4 added in the incineration, as the presence of excess of acid inhibits colour formation; 2 c.c. of the molybdate solution (2·5 p.c. in 5 *N* H_2SO_4) were replaced by 2 c.c. of a 2·5 p.c. solution in water.

The value of *k* for the pyrophosphate hydrolysis was about 390×10^{-3} reckoned with natural logarithms; reckoned with decadic logarithms this becomes 170×10^{-3}, which may be compared with Lohmann's value of about 250×10^{-3} [1928 *a*]. We usually found that 8–8½ min. heating was necessary for complete hydrolysis of the pyrophosphate, instead of the 7 min. given by Lohmann. For example, in Fig. 2, the phosphate set

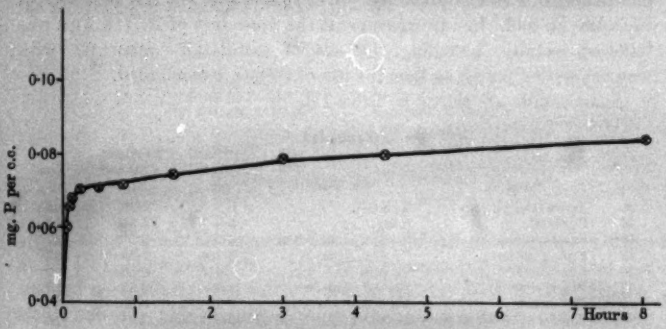

Fig. 3. Hydrolysis of adrenylpyrophosphate.
k for the pyrophosphate breakdown = 171×10^{-3},
k for the adenylic acid breakdown = 2×10^{-3} (using decadic logarithms).

free in the early stages by breakdown of adenylic acid may be found by extrapolating the steady slope of the curve backwards to the ordinate, and calling the phosphate value, where it cuts the ordinate, zero. If the phosphate values from this curve are subtracted from the experimental values, it is found that the pyrophosphate breakdown is complete in 8½ min. With the precipitates contaminated with hexose ester, this slowing of the pyrophosphate hydrolysis was particularly marked. In agreement with the results of Cori and Cori [1931–2*a*] with rat adenylpyrophosphate, it was found that, in the case of the fly, two-thirds of the adenylic acid phosphate was set free in 4 hours; the value of *k*, about 2×10^{-3}, is the same as the value calculated by Myrbäck and Euler [1931] from Levene and Jorpes' data [1929] for inosinic acid hydrolysis (using decadic logarithms).

As is well known, if the attempt is made to estimate the ribose in mammalian adenylpyrophosphate by the Hoffmann distillation method [1927], the yield of furfural is only a small proportion of that to be expected. Thus Embden and Schmidt [1929] report the production of only traces of furfural from rabbit adenylic acid; Steudel and Wohinz [1931] obtained 12 p.c. of the expected amount using inosinic acid from meat extract; Barrenscheen and Filz [1932] found 6 p.c. of the expected furfural was produced on distillation of rabbit adenylpyrophosphate. In our own experiments on fly muscle we obtained a slightly higher yield, but as a similar yield was obtained from frog adenylpyrophosphate, this fact is probably not of significance for the structure of the adenylic acid. In our experiments the procedure of Hoffmann was followed exactly, including the use of distillation apparatus with mercury-sealed joints, so that the use of rubber was avoided.

Some results are shown in Table III.

TABLE III.

Exp.	Material	Wt. used	Furfural expected mg.	Furfural obtained mg.	Yield %
28	Yeast adenylic acid	37·3 mg.	5·2	4·35	84
28	Furfural	5 mg.	5·0	3·85	77
28	Frog muscle	Ca ppt. from 5·3 g.	2·1	0·58	27·5
29	Frog muscle	Ca ppt. from 12 g.	3·75	1·38	37
20	Fly muscle	Ca ppt. from 6 g.	1·816	0·454	25
47	Fly muscle	Ba ppt. from 3·4 g.	1·36	0·35	25
50	Fly muscle	Ba ppt. from 4·6 g.	2·98	0·89	30
	Muscle adenylic acid	10·54 mg.	2·9	0·5	16

The expected furfural was calculated as the equivalent of half the pyrophosphate P, estimated in a sample of each muscle extract.

It was desired to compare, if possible, the result obtained for the adenylic acid of the muscle extracts by phosphate estimations with the result obtained by pentose estimations. It is obviously impossible to estimate the pentose content of muscle adenylic acid by Hoffmann's method; the method of Ferdmann [1933], in which 70 p.c. yields of furfural can be obtained from muscle adenylpyrophosphate by distillation in 6 *N* H_2SO_4 at 150° C., was not published till after this work was completed. An attempt was therefore made to estimate the pentose by means of the orcein-ferric chloride reaction of Bial. The estimation was carried out as described by Scheff [1924] for pentoses, except that a colorimeter was used instead of a spectrophotometer. The barium precipitate was dissolved in acid, and the volume was adjusted so that 1 c.c. should contain about 0·20 mg. adenylic acid (calculated from the figures found for pyrophosphate). 1 c.c. was placed in a dry tube and treated

with 4 c.c. of the Bial reagent. After 1 min. heating in a boiling water bath the tube was firmly stoppered (with a cork covered in tinfoil) and the heating was continued for another 9 min. After cooling, the solution was extracted by shaking, until completely colourless, with 5 c.c. amyl alcohol. After 10 min. the amyl alcohol layer was pipetted off and compared with a similar solution, prepared from muscle adenylic acid and containing 0·2 mg. adenylic acid per c.c. We are indebted to Dr Drury and to Dr P. Ostern for samples of muscle adenylic acid, in both cases made from mammalian muscle. The results obtained were unsatisfactory, being rather variable and always higher than was to be expected from the pyrophosphate results (Table IV).

TABLE IV.

Adenylic acid P (half pyro P) mg. per c.c. extract	Adenylic acid P (from pentose) mg. per c.c. extract
0·0170	0·0201
0·0170	0·0186
0·0170	0·0251
0·0177	0·0205
0·0177	0·0207

An attempt was also made to use the modification of Sumner [1923], as Dr Cori informed us in a private communication that this was the method successfully used in the work on the rat. With the fly preparations, however, this method also gave high results, and the colour produced was not the same in the unknown as in the standard. Further work must show whether these results are due merely to the presence of some factor in the fly muscle influencing the rate of furfural production, or whether they are due to some peculiar property of fly adenylic acid. It may be mentioned that with Scheff's modification of the Bial reaction, colour production was always markedly more rapid with the standard muscle adenylic acid solutions than with yeast adenylic acid solutions of the same concentration; after the 10 min. heating the colour developed in the yeast adenylic acid tubes was only about 75 p.c. of that in the muscle adenylic acid tubes.

THE HEXOSE ESTER FRACTION.

The total P content of the trichloracetic acid extract was shown to be considerably greater than the sum of the inorganic, phosphagen and adenylpyrophosphate P. As we shall see, this P probably belongs to hexosemonophosphate. The excess of P amounted, in two of the earlier

experiments, to 14·3 mg/100 g. and 16·4 mg./100 g. These values are probably rather low, as in these particular experiments precipitation had been carried out at *p*H 10, so that the values for adenylpyrophosphate subtracted from the total were too high. After precipitation at *p*H 7·4, the value of 19·4 mg./100 g. was found. In some later experiments the method of Cori and Cori [1931–2*a*] for the estimation of hexosemonophosphate (with one or two adaptations necessitated by the different material) was followed, and here again, as was to be expected, higher results were obtained.

In one such experiment the extract from 84 flies was neutralized to *p*H 7·4–7·6 with baryta. To the centrifugate after removal of the precipitate, four volumes of 97 p.c. alcohol (neutralized) were added, and the mixture was kept overnight at 0° C. In this way argininephosphoric acid and hexosemonophosphate are completely precipitated. Then the precipitate was centrifuged off and dissolved in 20 c.c. *N*/10 HCl; the barium was removed by addition of sodium sulphate solution followed by centrifuging. The argininephosphoric acid was then broken down by 15 min. heating in a boiling water bath. The free phosphate present after this treatment was estimated in one sample (*A*), as well as the total P in another sample (*B*).

The values obtained were:

A	...	...	25·7 mg. free P/100 g.
B	...	...	48·0 mg. „ „
Ester P		...	22·3 mg. „ „

The value *A* represents, of course, the phosphagen P, together with some preformed inorganic P which escaped precipitation by barium, on account of the low *p*H.

In another experiment the reducing value of the ester was compared with the P content as was done by the Coris. Three hundred flies were used; the precipitation was again at *p*H 7·4; in order to avoid any reduction by arginine, the argininephosphoric acid was hydrolysed (in *N*/10 acid for 30 min. at 100° C.) before the alcohol precipitation. After the hydrolysis, the solution was neutralized, and four volumes of neutralized alcohol were added. After standing for 3 hours the precipitate was centrifuged off; it was dissolved in acid, and the barium was removed by sodium sulphate solution. The volume was made up to 15 c.c. The free phosphate (*A*) present in 1 c.c. was estimated, and the total P (*B*) in another 1 c.c. sample. Two samples of 5 c.c. each were used for the estimations of reducing power by Hagedorn and Jensen's method, using the solutions described by Boyland [1928].

The phosphate estimations gave the following results:

A	... ...	24·9 mg. P/100 g.
B	... ...	43·5 ,, ,,
Ester P	...	18·6 ≡ 108 mg. hexose/100 g.

The reducing power of 5 c.c. was equivalent to 1·57 mg. glucose. For 100 g. the value would be 82·6 mg. As the reducing power of hexose-monophosphate is only 67 p.c. of that of glucose, the value must be multiplied by 100/67, in order that it may be compared with the hexose calculated from the P value. The result, 123 mg. hexose/100 g., is 14 p.c. high. This point will be further investigated, but at present it seems more reasonable to conclude that the presence of small amounts of other reducing substances (*e.g.* glutathione) [see Cori and Cori, 1931–2 *a*] is responsible rather than that a different hexosephosphate is present.

Discussion.

It will be interesting to compare the general distribution of soluble phosphorus found in the fly with the results given by Eggleton [see Hill and Kupalov, 1930] for the frog and by Cori and Cori [1931–2 *a*] for the rat.

Table V.

	P mg./100 g. muscle		
	Fly	Frog	Rat
Orthophosphate	25–35	18	115
Phosphagen	10–20	65	
Pyrophosphate	30–40	30	41
Esters with soluble Ba salts	20	5	7–10
Esters with insoluble Ba salts (adenylic acid of adenylpyrophosphate) ...	15–20	15	20·5

One striking difference is the low phosphagen content of the fly muscle. It is also considerably lower than that found in many of the other invertebrates, especially other insects by Schütze. It is, of course, possible that the value found after the treatment described is lower than that *in vivo*; that the argininephosphate P could not, however, even *in vivo* be higher than about 20 mg./100 g. is shown by experiments in which the total free arginine was estimated after hydrolysis of the phosphagen. The value obtained for the free arginine in such an experiment was 121 mg./100 g. the equivalent of which in P is only 21·5 mg./100 g.

Secondly, the fly muscle shows a high content of hexose ester, compared with the frog muscle, and with rat muscle removed under amytal anæsthesia. As Cori and Cori [1932–3] have pointed out, stimulation of excised rat muscles to contraction leads to increase in hexosemono-

phosphate content, but this increase soon disappears if the muscles return to rest. The low resting value of hexosemonophosphate P, 7–10 mg./100 g., was only obtained when the muscle was removed from the animal under amytal anæsthesia. The nervous discharges accompanying death by stunning, decapitation, etc., caused great rise in hexosemonophosphate content. Injection of epinephrine into the intact animal also led to the production of hexosemonophosphate in the muscles, and the rise might persist for some hours [Cori and Cori, 1931–2*b*]. Possibly the cold to which the intact flies are subjected acts as a stimulus to their nervous system, and the result is the production of some epinephrine-like substance. In the rat the phosphate necessary comes apparently from the free phosphate of the muscles themselves and of the blood. Both in their low phosphagen content and in their high ester content the fly muscles resemble the muscle of the frog's heart. The following values are from the paper by Clark, Eggleton and Eggleton [1931].

	mg. P/100 g.
Inorganic	11·6
Phosphagen	7·3
Pyrophosphate	11·2
Organic phosphates with insoluble Ba salts	11·3
Organic phosphates with soluble Ba salts	43·1

In the case of these intact, isolated, cold-blooded hearts there is no reason to suppose that the low phosphagen content and the high ester content found *in vitro* do not represent the state of affairs *in vivo*. Further work must decide the question for the fly.

Summary.

1. It has been shown that fly muscle contains argininephosphoric acid.

2. The distribution of the acid-soluble phosphorus has been quantitatively studied. The muscle contains an adenylpyrophosphate, probably the same as that in frog muscle, and a reducing ester, probably hexosemonophosphate.

3. The concentration of adenylpyrophosphate is very similar to that in the frog or rat; the argininephosphoric acid content is small and the hexose ester content high in comparison with the frog or rat.

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THE EXCRETION OF NON-METABOLIZED SUGARS BY THE MAMMALIAN KIDNEY.

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The search for renal function tests, which shall be based on sound physiological principles and not solely on empirical observation, has of recent years been conducted in two main directions. Firstly by attempts to form a true estimate of the volume of glomerular filtrate, and secondly by the study of phenomena which are presumably based on the activity of the renal tubule cells.

It is clear that once the true volume of glomerular filtrate is known, the rôle of the tubule cells will immediately be more evident, and it is accordingly of great importance that any claims to have found a method of measuring glomerular filtration rate should be critically examined.

Evidence continues to accumulate, from the work especially of Richards and his associates [1933], that the composition of the filtrate is the same as that of an ultrafiltrate of plasma. Hence, if any substance can be found which enters the tubule lumen by filtration through the glomerulus, and in its passage down is neither added to by secretion or diffusion through the tubule cells, nor reduced in amount by reabsorption in these cells, then that substance can be employed to indicate the volume of glomerular filtrate. The "clearance" for this substance must be identical with the volume of glomerular filtrate [Cope, 1931].

After the abandonment of urea for this purpose by Cushny, there remained two substances which, it was felt, might still fulfil the required conditions. These two were creatinine and inorganic sulphate. But since it was shown that these substances had entirely different clearance values in the human kidney, that of the former being about three times as great as that of the latter, it was evident that one at least of them would have to be discarded [Cope, 1932; Hayman and Johnston, 1932].

Rehberg and the Scandinavian workers generally have persistently upheld their claims for creatinine, although the evidence they bring in support is relatively slender. Hayman and Johnston [1932] attributed the wide discrepancy between the sulphate and creatinine clearances to a tubular reabsorption of the former rather than to any secretion of the latter. Cope [1932], however, felt that the available evidence was entirely inadequate to decide between the apparently rival claims of these substances.

In 1932, however, new substances for consideration were introduced by Jolliffe, Shannon and Smith, *i.e.* the non-metabolized sugars xylose and sucrose. The evidence brought forward in favour of these sugars was much stronger than that which had been accumulated for creatinine.

The main points may be briefly summarized as follows:

(1) Xylose and sucrose have the same clearance value when this is determined simultaneously for both in the mammalian kidney [Jolliffe, Shannon and Smith, 1932].

(2) Under the action of phlorrhizin glucose excretion increases until the glucose clearance comes to equal that already shown by xylose and sucrose [Jolliffe, Shannon and Smith, 1932].

(3) Xylose is readily excreted by fish whose kidneys possess glomeruli, but only in traces by those with aglomerular kidneys [Jolliffe, 1930; Clarke and Smith, 1932].

(4) Glucose is excreted by glomerular but not by aglomerular kidneys in fish under the action of phlorrhizin [Marshall and Grafflin, 1928; Marshall, 1930].

(5) After phlorrhizin the glucose clearance comes to equal the xylose clearance in fish with glomerular kidneys [Jolliffe, 1930; Clarke and Smith, 1932].

(6) Similar phenomena are also presented by the frog's kidney [Marshall, 1932].

These observations are readily explained only if it is supposed that all three sugars are normally filtered through the glomeruli and do not enter the tubule lumen through the tubule cells except in traces, and that normally glucose is reabsorbed by an activity of the tubule cells which can be inhibited by administration of an adequate dose of phlorrhizin.

If this be admitted, then certain further results of these workers must be held to show that creatinine is excreted in part at least by the tubule cells. For Shannon, Jolliffe and Smith [1932] found the normal creatinine clearance in dogs to be 15–50 p.c. higher than that for xylose

(or sucrose), while they claimed that after phlorrhizin the creatinine clearance fell to the same level as that for xylose, sucrose and glucose. And Clarke and Smith [1932] showed further that in the dogfish also creatinine is excreted more readily than xylose, a fact which they interpret as evidence of tubular secretion of the former.

In this respect also, certain recent experiments by Höber [1932] are of interest. He found that when creatinine was supplied to the glomeruli of the frog's kidney by perfusion into the aorta, it was readily excreted. But when it was supplied by the portal vein to the tubules it was also excreted though in greatly diminished amount, a fact which he considers as evidence that, although most of the creatinine is eliminated by glomerular filtration, some leaks through the tubule cells.

It is clear, therefore, that the work of Smith and his associates is of such importance as to require early confirmation.

White and Monaghan [1933] attempted to repeat the observations on the behaviour of creatinine and glucose during their excretion under the action of phlorrhizin, but found that in only one experiment out of thirteen did the glucose clearance rise to equal the creatinine clearance.

The present work also was undertaken with a view to determining to what extent the results of Smith and his co-workers could be confirmed.

Unfortunately facilities were not available for the use of dogs, and accordingly rabbits had to be employed, so that it is not possible to compare strictly the results here presented with the findings of Smith and his associates. Those that agree may be regarded as confirmatory, but those that differ may possibly be due to the use of a different experimental animal.

Methods.

Creatinine in urine and plasma has been estimated by the standard methods with alkaline picrate, taking the usual precautions as to purity of picric acid and similarity of concentration of standard and unknown solutions. Plasma concentrations were always determined before the corresponding urine values, and were throughout done on 1 in 10 Folin-Wu filtrates. In the earlier experiments an ordinary Duboscq colorimeter was employed, but in the later a Leitz universal compensating colorimeter was used. With this instrument the insertion of the green light filter greatly increased the convenience and accuracy of the colour comparison.

Sucrose in plasma. The principles of the methods used by Jolliffe, Shannon and Smith [1932] have been employed as regards incubation with sucrase at 40° C. for 1 hour at *p*H 4·0–4·5 (to bromphenol blue) and

subsequent neutralization with sodium hydroxide (to phenolphthalein). For the actual sugar estimation the titrimetric micro-method of Shaffer-Hartmann as modified by Somogyi [1926] was considered preferable to a colorimetric method, and recoveries for both glucose and sucrose were within 2 p.c. of theoretical in known solutions. Duplicate analyses made in all cases were in good agreement.

Sucrose values were obtained by subtracting the plasma glucose figures from those for glucose + sucrose after inversion with sucrase. When only sucrose figures were required Folin-Wu filtrates were used, but when other sugars were also being determined the copper filtrates of Somogyi [1931] were used. Early difficulties in preparing clear filtrates from rabbits' plasma by this method were found to be due to the fact that more copper sulphate was required than for human blood to bring the final *p*H of the filtrate to about 6·0. In the proportions of 1·5 c.c. of 5 p.c. copper sulphate and 1·0 c.c. of 6 p.c. sodium tungstate to 1·0 c.c. plasma and 6·5 c.c. of water, satisfactory filtrates were obtained.

Sucrose in urine was determined by essentially the same procedure using suitable dilutions of the urine, usually 1 in 100 or 200. Somogyi copper filtrates were usually employed, and invariably so when other sugars were also being determined. When sucrose alone was present the blank values obtained before inversion were practically negligible except when creatinine concentrations were high, for copper precipitation only removed about 60 p.c. of the creatinine in trial experiments. Using the Shaffer-Hartmann-Somogyi micro-method a 1 p.c. solution of creatinine in urine gave a reduction equivalent to about 300 mg. of glucose, and after the precipitation this was reduced to 130 mg. But by using the same analytical method for plasma and urine and diluting the urine to a concentration similar to that of the plasma, the resultant small error was practically eliminated, as Shannon, Jolliffe and Smith [1932] have pointed out. The recoveries of sucrose in urine were within 2 per cent. with satisfactory agreement of duplicates.

Sucrase was made by the method of Morrow [1927], after submitting the yeast to a preliminary "enreicherung" before autolysis according to the recommendation of Willstätter, Lowry and Schneider [1925]. 0·2 c.c. of the resultant clear solution was ample for a single analysis, and the blank due to the enzyme solution was small.

Glucose in both plasma and urine was always determined from the difference in reducing activity before and after fermentation with washed yeast of copper filtrates according to the procedure of van Slyke and Hawkins [1929].

Xylose. For a generous supply of xylose we are indebted to the United States Chamber of Commerce, who also supplied full details of the methods to be used for its purification. It was estimated in both plasma and urine as unfermentable reducing substance in copper filtrates. The finding of Jolliffe, Shannon and Smith [1932] that, during the fermentation, yeast absorbs about 13 p.c. of the xylose, has been confirmed for filtrates containing about 10 mg./100 g. of the sugar. With our yeast it has tended to increase with higher concentrations, reaching 16 p.c. with 50 mg./100 g., and falling to about 10 p.c. in solutions with 5 mg. representing filtrates of a blood with 50 mg./100 g. To avoid complications, however, the observed non-fermentable reductions have been multiplied by 100/87 to obtain the true xylose figures. The Shaffer-Hartmann-Somogyi micro-method with 15 min. heating gives a reduction corresponding to only 95 p.c. of that shown by the same concentration of glucose. But since this factor enters equally into all analyses in which xylose is present and does not affect the calculation of the clearance, it has not been applied. The phlorrhizin used in some experiments must affect the reduction figures. With the sugar method used 100 g. phlorrhizin gave a reduction equivalent to 13 mg. glucose. Since this is apparently not removed either by the copper precipitation or the yeast treatment, it will be estimated in the xylose fraction. But since xylose is present in amount three to four times that of phlorrhizin, the reduction due to the latter will not exceed about 4 p.c. of the former. Hence, unless the two substances have widely different clearances, the phlorrhizin will not greatly affect the estimation of the xylose clearance. Nevertheless, it is probable that the xylose analyses are rather less accurate than those of the other sugars.

Experimental.

Male rabbits were used throughout. 40–100 c.c. of an aqueous solution containing such quantities of sucrose, xylose and creatinine as would give approximately the desired plasma concentrations were injected subcutaneously. About an hour was allowed for absorption before collection of urine samples was begun. All urine collections were made by a soft rubber catheter, and although the bladder was not washed out, it is not believed that the volume of residual urine was large. Blood samples were collected at the middle of each urine period from an ear vein, being allowed to drip directly into a centrifuge tube containing potassium oxalate. The samples were centrifuged immediately, and from the plasma

Folin-Wu or copper filtrates were prepared within 2–3 hours. These filtrates, when not analysed the same day, kept well in the cold store overnight.

In no experiment was any anæsthetic used, the animals remaining placid and normal throughout.

When phlorrhizin was administered, it was given by subcutaneous injection dissolved in 1·5 p.c. sodium bicarbonate in a uniform dose of 1 g. to rabbits averaging 2·5–3·0 kg. in weight.

Results and discussion.

A comparison of the sucrose and creatinine clearances was first entered upon before the work of Shannon, Jolliffe and Smith [1932] was published. Sucrose was used in preference to xylose because these workers had previously claimed that the two sugars behaved similarly and were presumably interchangeable. Sucrose analyses were apparently rather more accurate than those with xylose, and further, at the time this work was done, an adequate supply of xylose was not available.

It soon became apparent that the creatinine clearance is practically always somewhat higher than that for sucrose during the same period. In this we therefore fully confirm the published results of the above workers.

In this series a wide range of clearance values was encountered, owing mainly to the fact that two of the rabbits were fed for some weeks on a diet of bran and oats, which was associated with a serious fall in the clearances, a fall which did not occur on a diet of green vegetables.

In Fig. 1 the observed creatinine clearances are plotted against the corresponding sucrose clearance figures. It will be seen that there is a definite proportionality between the two, although more scattering exists than is to be accounted for by experimental errors. The dotted line in the figure is the line of equality of the two clearances. In all cases but one the points lie above this line, the creatinine clearances therefore exceeding the sucrose clearances.

Now the creatinine clearance is the basis of Rehberg's test for renal function, and is extensively used for clinical purposes in Scandinavia and to a less extent elsewhere. It was accordingly felt that, if this test is to justify its use for scientific purposes, it should be feasible to use it to examine the possibility that, in the excretion of substances of this type, there exists an essentially linear relationship between plasma concentration and rate of excretion, but that this is frequently obscured by

spontaneous and irregular variations in general renal activity. A case has indeed been recorded of one human subject in whom, under certain conditions, such a relation did exist for creatinine [Cope, 1931], but this does not appear to be the general experience.

We have therefore used the creatinine clearance as a measure of general renal activity whilst studying the excretion of sucrose at varying plasma concentrations. In such work it is unnecessary to assume the correctness of Rehberg's contention that creatinine measures the glomerular filtrate, and it is for this reason that we prefer to use the term

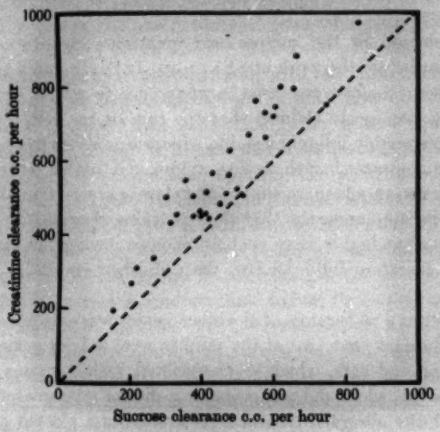


Fig. 1.

general renal activity. In this group of experiments plasma creatinine concentrations have been kept within the relatively narrow limits of 7–14 mg./100 g. in order as far as possible to minimize any possible influence of varying creatinine concentration. Sucrose concentrations have meanwhile been varied from 20–220 mg./100 g. in the plasma. The observed sucrose excretions have been corrected for any variations in this general renal activity as judged by the creatinine clearance, assuming that full activity is indicated by a creatinine clearance of 700 c.c. per hour. Thus, if the observed creatinine clearance in a given period is only 600, the observed sucrose excretion during this period has been multiplied by 7/6, since the kidney was then only exerting 6/7 of its full activity.

In Fig. 2, then, the plasma sucrose concentration has been plotted against the sucrose excretion rate corrected to a constant general renal activity corresponding to a creatinine clearance of 700 c.c. per hour. The suspected linear relation is at once revealed.

If now the conditions are reversed, and plasma creatinine concentration is varied over a wider range, whilst the sucrose clearance is used as an index of general renal activity, the observed creatinine excretion rate can similarly be corrected for variations in this activity in like manner. In Fig. 3 plasma creatinine concentration has been plotted against the observed excretion rate after this has been corrected to a constant renal activity corresponding to a sucrose clearance of 500 c.c. per hour. With

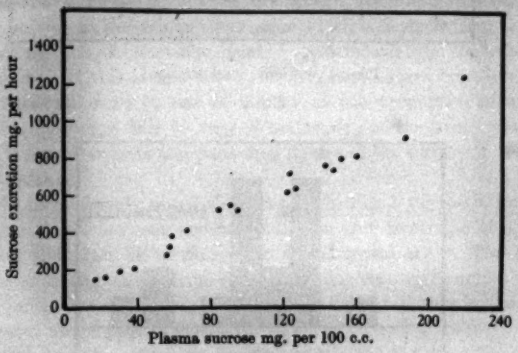


Fig. 2.

the exception of one, all points fall close to a straight line and a linear relationship is again demonstrated. This figure is of interest also from another point of view. The objection is frequently raised against experiments with creatinine that the existence of this substance in normal plasma is in doubt, and that therefore the total chromogenic substance is no measure of the amount of available creatinine. If this were an appreciable factor in determining the excretion of creatinine we should expect its effect to be increasingly evident as the amount of exogenous or added creatinine in the plasma is diminished. Fig. 3 shows, however, that this is not the case. Creatinine excretion has been found to be no less regular when the "apparent" creatinine in plasma is low than when it is high. Yet at the low concentrations the excreted creatinine is presumably derived mainly from the unknown plasma chromogen.

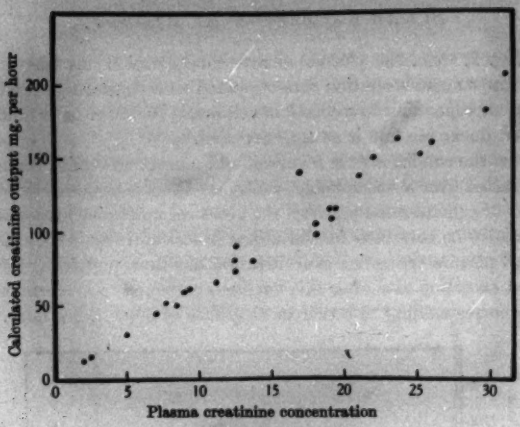
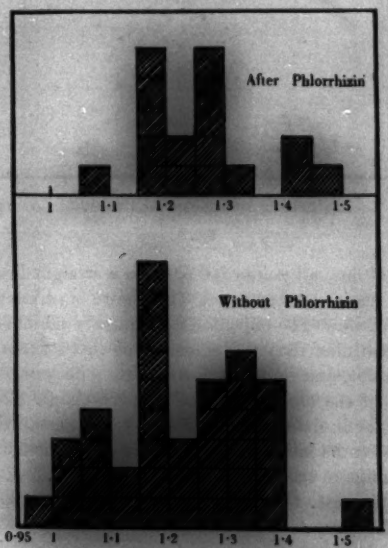


Fig. 3.



Distribution of values of the ratio
creatinine clearance/sucrose clearance

Fig. 4.

This fact, and also the experiments previously reported on a human subject [Cope, 1931], show that in practice no significant error is introduced in work of this kind by assuming that, so far as the kidney is concerned, practically all the available chromogen is treated as creatinine. Whilst we are willing to accept the view that the chromogen of normal plasma does not behave chemically like creatinine, we feel nevertheless that when the plasma concentration is artificially raised, no significant errors are introduced by regarding the whole as creatinine. White and Monaghan [1933] have taken as the creatinine content of plasma in their experiments the observed value minus the value obtained on a normal plasma before administration of creatinine. This method is only permissible if, at the same time, the urine is dealt with in a similar way. That portion of the urinary creatinine which is derived from the normal plasma chromogen must also clearly be subtracted from the total urinary creatinine. If this is not done, the resultant figures for the creatinine clearance will tend to rise to infinity as the proportion of exogenous plasma creatinine falls to zero. Whether or not the urine was so dealt with is not clear from the published figures of the results of White and Monaghan.

We have already noted the fact that in Fig. 1 the deviations from a linear relation between sucrose clearance and creatinine clearance are greater than can be attributed to experimental errors. The extent of variation of the value of the ratio creatinine clearance/sucrose clearance is an indication of this (Fig. 4), for if both linear relations held accurately the ratio would remain constant.

Now Smith and his co-workers explain the fact that the creatinine clearance exceeds that for sucrose by supposing a secretion by the tubules of that portion of the excreted creatinine responsible for the difference between the two clearances. But since this explanation, though a probability, is not yet a certainty, we prefer to allude to this extra creatinine by the less commital term "excess creatinine clearance."

If we suppose that the excretion of this extra creatinine is brought about by a process in the kidney different from that responsible for the excretion of the sucrose on the one hand, and an amount of creatinine corresponding to the same clearance as the sucrose clearance on the other, then it is evident that this process is of very variable activity and may be responsible for from 0 to 30 p.c. or more of the total quantity of creatinine excreted.

Attempts to correlate the variations in the excretion of this fraction, the "excess creatinine clearance," with other factors which might be

expected to influence it, if it be a tubular secretion or diffusion, have not been successful. It apparently bears no relation to the absolute value of the clearance. Nor do the actual concentrations of creatinine in the plasma or the urine seem to be determining factors. This lack of correlation with the plasma concentration is also evidence that the variations in this "excess creatinine clearance" are not attributable to discrepancies between the "apparent" and the "true" creatinine of these plasmas.

The possibility that the variations are rather due to changes in sucrose excretion cannot be ignored, but here again no influence of the concentration of sucrose in plasma or in urine could be detected.

Attempts to influence the value of the "excess creatinine clearance" by urea have also failed, no definite effect having been noted.

We therefore have no real clue as to the origin of this "excess creatinine" or to the reasons for its variations. In their very irregularity they are perhaps most in keeping with the view that this process is one of secretion, and we feel inclined provisionally to accept this explanation in the absence of further evidence.

Now Shannon, Jolliffe and Smith [1932] have claimed that under the influence of phlorrhizin the creatinine clearance falls and becomes equal to the xylose clearance, so that this "excess creatinine clearance" disappears. In our experiments using sucrose in place of xylose it has not been possible to confirm their findings. The values of the ratio creatinine clearance/sucrose clearance obtained in a number of such comparisons are shown in Fig. 4. It will be seen that the distribution is not significantly different from that obtained in the absence of phlorrhizin. There has appeared no constant tendency for the value of this ratio to approach unity, although in most cases the glucose excretion indicated a complete phlorrhizination. Typical figures obtained are shown in Table I.

If, however, the creatinine clearance is compared with the xylose clearance a definite fall in the ratio between the two can always be noted. This discrepancy can be accounted for by the fact that in our experiments the xylose clearance has been found to be consistently below that for sucrose in the absence of phlorrhizin, the value of the ratio sucrose clearance/xylose clearance varying between 1·17 and 1·39, with a mean of 1·30. Phlorrhizin has brought this ratio down to unity within the limits of experimental error, the mean value of the ratio then becoming 0·98. We are not prepared to say definitely whether this is due to a fall in the sucrose clearance or to a rise in that of xylose, but are inclined to the view that the sucrose clearance falls to the xylose level.

TABLE I.

Exp.	Urine vol. c.c. per hr.	Sucrose			Xylose			Glucose			Creatinine			Ratios			
		Plasma	Urine	Clearance	Plasma	Urine	Clearance	Plasma	Urine	Clearance	Plasma	Urine	Clearance	Cr./Su.	Glu./Su.	Xy./Su.	Cr./Xy.
1	10·3	193·6	4179	222	—	—	—	165·2	—	—	21·9	656	308	1·39	—	—	—
	11·8	203·6	3800	221	—	—	—	182·2	2440	158	23·2	510	200	1·18	0·71	—	—
	10·0	180·4	3283	182	—	—	—	192·4	3605	187	23·6	454	192	1·06	1·03	—	—
2	7·06	250·0	9403	266	—	—	—	194·9	—	—	29·7	1410	335	1·26	—	—	—
	7·78	139·5	4085	227	—	—	—	198·6	5428	212	17·2	622	281	1·24	0·94	—	—
	5·16	100·6	3621	186	—	—	—	209·4	5915	146	14·2	610	221	1·19	0·78	—	—
	5·03	76·3	3602	238	—	—	—	156·1	5678	183	11·8	705	300	1·26	0·77	—	—
3	18·5	146·1	3248	411	178·5	3055	316	117·8	—	—	32·0	975	564	1·37	—	0·77	1·78
	14·7	83·7	2088	368	79·8	1986	367	125·2	2684	315	21·0	614	430	1·17	0·86	1·00	1·17
	14·1	63·8	1715	380	56·3	1590	399	127·3	3121	346	16·6	645	547	1·44	0·91	1·05	1·37
4	35·4	118·4	1736	519	156·2	1640	372	128·3	—	—	29·7	505	602	1·16	—	0·72	1·62
	18·8	114·4	2195	360	114·0	2300	379	115·5	2164	353	23·9	615	484	1·34	0·98	1·05	1·27
	15·7	70·7	2186	486	73·5	2100	450	121·3	2503	324	17·9	710	622	1·28	0·66	0·92	1·38
5	25·0	116·0	1814	392	137·0	1690	308	134·5	—	—	17·0	296	435	1·11	—	0·79	1·41
	14·8	93·6	2466	391	85·6	2380	412	118·3	3165	396	13·2	429	480	1·23	1·01	1·05	1·16
	10·6	58·9	2327	419	56·2	2040	385	125·7	3045	257	9·7	450	492	1·18	0·61	0·92	1·28
6	24·0	100·2	2200	526	102·5	1905	446	99·0	—	—	12·2	278	546	1·04	—	0·85	1·22
	13·4	47·6	1796	505	44·5	1810	545	103·5	3769	489	6·2	290	628	1·24	0·97	1·08	1·15
	11·1	28·7	1282	496	29·9	1374	510	113·3	5147	503	5·0	287	638	1·28	1·01	1·03	1·25

Note. A dose of 1 g. of phlorrhizin was administered after period 1 in each experiment. Period 2 began about 1 hour after this injection, except in Exp. 1 in which it began immediately after the injection.

Nevertheless, the creatinine clearance has never fallen to the xylose level even during the periods when phlorrhizin action was complete as judged by the glucose excretion, the mean value of the ratio creatinine clearance/xylose clearance being then 1·21 compared with 1·55 in the absence of phlorrhizin. In this our results thus agree better with those of Poulsson [1930], and of White and Monaghan [1933] than with those of Shannon, Jolliffe and Smith [1932].

That during the action of phlorrhizin the glucose clearance rises to equal the sucrose and xylose figures we have been able fully to confirm. The mean of the maximum values of the ratio glucose clearance/sucrose clearance reached in each of seven experiments was 0·97.

We have, in these experiments, been dealing with plasma creatinine concentrations which are on the whole considerably lower than those used by Shannon, Jolliffe and Smith. The possibility that herein lay the cause of the failure to reduce the creatinine clearance to the xylose and sucrose level could not be ignored. It seems unlikely that this is the case however. These workers have observed their effect with plasma creatinine concentrations below 20 mg./100 c.c. Moreover, they state that in dogs the non-creatinine chromogenic substance never exceeded 0·5 mg./100 g., so that with plasma creatinine level above 10 mg./100 g. the resultant error is small. The discrepancy we find between the creatinine and xylose clearances under phlorrhizin averages about 20 p.c. and would appear therefore to be much too large to be accounted for in such a way.

We find ourselves quite unable to account for these phenomena of the excretion of the sugars except along the lines of the views held by Smith and his associates. That is, that phlorrhizin diminishes or abolishes the permeability of the tubule cells, so that on the one hand the reabsorption of glucose is completely inhibited, and on the other the diffusion or secretion of creatinine and possibly also of sucrose is greatly reduced. That we have been unable to abolish completely this apparent diffusion of creatinine, and in this respect differ from the Bellevue Hospital workers, does not seriously affect the conclusions which one feels compelled to draw. Nor does our finding that in the absence of phlorrhizin the sucrose clearance exceeds that for xylose.

It is not difficult to believe that, in the phlorrhizinized kidney, some permeability of the tubules for creatinine is still present.

But whether or not this explanation of the facts be accepted, and it is difficult to put forward any other, it would seem quite certain that the figure representing the xylose clearance has a real meaning in the kidney. The same figure can be arrived at by a study of the excretion of either

sucrose or glucose under the influence of phlorrhizin. A rough approximation to it can be obtained from the creatinine clearance under phlorrhizin or the sucrose clearance without phlorrhizin.

Further support for the importance of this figure is contained in the succeeding paper, in which it is shown that precisely the same value can be arrived at by the use of an entirely independent substance, having widely different chemical properties [Cope, 1933]. So far as we are aware no other value is known for the kidney which can be arrived at in even two ways, without irreversible damage to that organ.

We feel, therefore, that the phenomena presented by the excretion of these substances well merit further investigation, and that they offer great hope for the development of a renal function test along rational lines.

Summary.

The excretion of the sugars xylose and sucrose has been compared with that of glucose after phlorrhizin, and with creatinine with and without phlorrhizin in the rabbit's kidney.

In confirmation of previous workers the clearances for all three sugars have been found equal after phlorrhizin.

In the absence of phlorrhizin the sucrose clearance was about 30 p.c. higher than the xylose clearance.

The creatinine clearance may be from 0 to 50 p.c. higher than the sucrose clearance, but is never lower. This difference is not appreciably affected by phlorrhizin.

Creatinine clearance averages 55 p.c. above xylose clearance, but falls to only 20 p.c. above it after phlorrhizin. It has never been found equal to the xylose clearance.

Creatinine clearance is roughly proportional to the sucrose clearance over a wide range of clearance values. The ratio between the two is not appreciably influenced by the concentrations of either in plasma or urine, nor by the action of urea.

If allowance be made for varying renal activity, an approximately linear relation can be found between plasma concentration and rate of excretion for both creatinine and sucrose.

The interpretation of these findings is considered.

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THE EXCRETION OF CYANOL BY THE MAMMALIAN KIDNEY.

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The interesting experiments forming the basis of claims recently put forward for the sugars xylose and sucrose as indicators of glomerular filtration rate in the kidney have been in part confirmed and discussed in the previous paper [Cope, 1933].

It became of interest also to compare the excretion of these sugars with that of another substance of quite different type which had also been claimed to behave similarly in the kidney—the dye "Cyanol extra."

The experimental phenomena presented by xylose and sucrose have already been stated elsewhere and need not be further repeated here, but it is convenient briefly to summarize the evidence relating to cyanol.

Cyanol belongs, according to Höber, to the group of highly diffusible, lipoid insoluble acid dyestuffs. It is readily excreted in the urine of most animals after subcutaneous or intravenous injection. But when injected into the blood stream of the fish *Lophius piscatorius*, whose kidney has no functional glomeruli, it appears only in traces in the urine, although phenol red injected at the same time is readily excreted [Höber, 1930].

Similarly, when the glomeruli of another fish, the sculpin, are rendered inactive by several doses of phlorrhizin, injected cyanol does not appear in the urine in appreciable amount [Marshall and Grafflin, 1932].

Again, when supplied to the tubules of the frog's kidney by perfusion through the portal vein, it is excreted in only small amount at low concentration, although when perfused by the aorta through the glomeruli, it is much more rapidly excreted and more highly concentrated in the urine [Schulten, 1925]. In view of these and other similar results Höber considered it was proved that cyanol is a purely glomerular excretory product, not being appreciably influenced by the tubule cells.

Descombes [1932] accordingly attempted to show that both cyanol and creatinine behave in identical manner in the mammalian kidney, his purpose being to obtain further support for Rehberg's contention [1926] that creatinine also is excreted only by the glomeruli. The conclusions which he draws are, however, scarcely justified by the results which he presents. No blood analyses were made, and it was presumably assumed without experimental proof, that after intraperitoneal injection both were absorbed at equal rates into the blood stream. Further, as Descombes himself freely admits, the technical difficulties of estimating both creatinine and cyanol simultaneously in the same solutions are very great owing to the interference of the blue colour with the orange of the Jaffe reaction for creatinine. He found, however, that there was a fairly good parallelism between the rates of excretion of cyanol and creatinine, but his conclusion that this indicates a similarity of excretory mechanism for both we cannot accept on his evidence.

Frey also [1932] has, in a few experiments, used cyanol as a measure of glomerular filtration rate, but adequate comparisons with other methods were not made. He obtained, in one experiment on a rabbit under urethane, figures which indicate a cyanol clearance in two periods of 168 and 300 c.c. per hour. Such figures are rather below those usually obtained by the use of sucrose or xylose, but this may well be due to the effect of the urethane.

In the work here to be presented cyanol excretion has been compared in rabbits with that of xylose, sucrose and glucose.

Methods.

The methods used in the sugar analyses have been discussed elsewhere [Cope, 1933] and were used here also without modification. Somogyi copper filtrates of both blood plasma and diluted urine were used in all cases.

The method used to determine the concentration ratio for cyanol calls for explanation however. For a supply of this dyestuff we are indebted to the generosity of Messrs Bayer Products, who assure us that it is similar to that used by Prof. Höber himself.

The dye dissolves freely in water to give a deep blue solution. Spectroscopically examined a weak solution shows a well-marked absorption band in the region 590–620 $\mu\mu$. It differs markedly from hæmoglobin in showing no significant absorption in the blue. This fact has been made use of in estimating cyanol in plasma. It was not found

possible to obtain plasma samples from rabbits, which were so free from hæmolysis or slight yellow tinge that they could be accurately compared in a colorimeter against an aqueous cyanol standard. The yellow tinge of the plasma has accordingly had to be compensated by the insertion of a cyanol-free plasma on the other side of the colorimeter. In doing this, the Leitz universal compensating colorimeter has been found to be of the greatest value.

It has been employed as follows: After obtaining a satisfactory centred light source, the cyanol-containing plasma sample is placed, without further treatment than good centrifuging, and without dilution, into a micro cup on the left side of the middle stage of the colorimeter. A cyanol-free plasma, obtained preferably from the same rabbit before it has received the cyanol injection, is placed in the corresponding right cup. In the top stage, where micro cups are not necessary, water is placed in the left-hand cup and a suitable cyanol standard on the right side. The coupled plungers of this upper pair of cups are then lowered to the bottom so that the light passes through the two plasmas only. The bottom stage of the colorimeter is left empty. The blue light filter (No. 2) which transmits mainly at 450 $\mu\mu$ is then inserted in the system. Now, with the left-hand blue plasma adjusted to a definite depth, usually 5 or 10 mm., differences in the intensity of the two halves of the visual field are due partly to opalescence but mainly to the yellow colour of the plasma itself. The right-hand cyanol-free plasma is now adjusted until the light intensity is equal on the two sides of the field. Next, without moving either plasma, the light filter is changed to a red (No. 9), transmitting at a wavelength of about 600 $\mu\mu$. The field again become unequal owing to the light absorption of the cyanol in this part of the spectrum, and then, still using the same red filter, the upper coupled plungers are raised until equality of the fields is restored, and the comparison between plasma and aqueous cyanol standard is thus made.

This procedure, which is much simpler in practice than in description, has appeared to give quite satisfactory results even when hæmolysis was sufficient to give a perceptible greenish tinge to the cyanol-containing plasma.

Actually, in these experiments, determination of the cyanol concentration ratio was of greater importance than a knowledge of the absolute cyanol concentrations. Hence, in order to minimize experimental errors, the corresponding urine suitably diluted was used in the upper cups, in place of a standard of known concentration. In this way the cyanol concentration ratio has been measured directly. The absolute concentration

in the urine was found by comparing a diluted urine sample with an aqueous standard containing 1 mg. cyanol per 100 c.c. Plasma cyanol concentrations were then calculated from the urine concentration and the concentration ratio.

Results and discussion.

Preliminary comparisons of the cyanol clearance with that of sucrose revealed that although they are of similar magnitude, the identity is not complete. The sucrose clearance tends to be some 25 p.c. higher than the cyanol clearance.

Urine vol. c.c. per hr.	Sucrose			Cyanol		
	Plasma	Urine	Clearance	Conc. ratio	Clearance	Cyanol / Sucrose
26·8	137·4	2781	543	15·3	410	0·75
21·1	136·7	3520	545	25·1	530	0·97
7·2	149·9	12507	601	73·0	525	0·875
6·9	140·3	12203	599	69·0	475	0·795
					Mean	0·85

But since it had been previously found that xylose has a clearance some 20 p.c. lower than sucrose in the absence of phlorrhizin, a direct comparison of xylose with cyanol was evidently likely to give a closer correspondence between the two clearances. This has accordingly been made, and although occasional deviations have occurred, the tendency for the two clearances to be identical has been definite. In a series of thirteen such comparisons the value of the ratio cyanol clearance/xylose clearance has been within the limits of 0·975 and 1·03 in ten, and in the remaining three was 0·85, 0·83 and 0·79.

Table I. Comparison of xylose and cyanol clearances.

Urine vol. c.c. per hr.	Xylose		Cyanol		Clearances		Ratio Cyanol / Xylose
	Plasma	Urine	Plasma	Urine	Xylose	Cyanol	
45·6	133·0	1170	8·6*		401	392	0·98
21·2	116·0	2245	20·0*		411	424	1·03
18·0	140·0	2180	2·4	36·3	280	272	0·975
11·3	115·5	3950	2·0	66·6	386	377	0·975
9·33	139·0	5150	3·16	117·0	345	345	1·00
10·2	131·5	5025	2·67	100·0	390	382	0·98
8·13	100·4	3723	2·96	109·0	301	290	0·995
1·96	89·7	7019	2·92	195·0	153	131	0·856
16·8	85·3	1819	2·76	48·8	358	297	0·83
10·6	68·1	2461	1·81	64·5	384	378	0·98
						Mean	0·96

* Concentration ratio.

TABLE II. The effect of phlorrhizin on the cyanol clearance.

Exp.	Urine vol. c.c. per hr.	Xylose		Glucose		Cyanol		Clearances			Glucose / Xylose	Cyanol / Xylose
		Plasma	Urine	Plasma	Urine	Plasma	Urine	Xylose	Glucose	Cyanol		
					Commenced 1 hour after 1 g. of phlorrhizin.							
1	19·1	155·0	3220	104·1	2327	9·48	76·4	397	426	154	1·07	0·39
	15·7	143·0	2910	111·3	2463	9·51	88·1	320	347	145	1·08	0·455
2	10·0	153·5	6420	149·4	Trace	3·32	109·0	418	—	328	—	0·79
					1 g. of phlorrhizin subcutaneously.							
	18·3	115·0	3678	147·2	3495	2·99	41·4	583	433	252	0·74	0·43
	15·8	77·3	2563	145·4	4816	2·68	32·5	525	523	191	1·00	0·36
3	7·95	128·3	5266	—	Trace	2·67	110·0	326	—	327	—	1·00
					1 g. of phlorrhizin subcutaneously.							
	13·65	120·2	3028	146·1	2903	3·86	58·0	344	272	205	0·79	0·60
	12·8	98·2	2451	151·9	3750	4·05	44·0	320	316	139	0·99	0·43
4	12·3	174·0	5400	—	Trace	3·67	111·0	382	—	373	—	0·98
					1 g. of phlorrhizin subcutaneously.							
	17·5	135·0	3230	150·6	2818	3·39	47·7	419	327	246	0·78	0·59
	18·0	103·8	2175	166·2	3810	3·31	32·8	377	380	178	1·01	0·47

This marked tendency of the value of the ratio to be unity would seem too great to be attributed to chance, and it appears justifiable therefore to attribute the three discrepant values to the existence of some interfering process which is not usually present.

On attempting to extend this comparison to include glucose in the phlorrhizinized kidney, entirely unexpected results were obtained. For while the glucose clearance rose to equal the xylose figure, that for cyanol fell very considerably to a value much below the other two. Its behaviour therefore was practically the opposite of that of glucose. Taking only those values obtained during full phlorrhizinization, as indicated by the value of the glucose clearance/xylose clearance ratio, the mean value of the ratio cyanol clearance/xylose clearance is then only 0·42 as compared with a mean of 0·95 for all the thirteen values of the same ratio obtained in the absence of phlorrhizin.

The inhibitory effect of phlorrhizin on cyanol excretion is thus very large, and is in fact readily apparent by simple inspection of the excreted urines.

In Fig. 1 it is compared with the behaviour of the sugars and creatinine towards phlorrhizin.

The interpretation of these findings is not easy, nor is it rendered any easier by the most recent work of Höber and Meirowsky [1932] on the excretion of cyanol in the frog's kidney.

The correspondence of the cyanol and xylose clearances in the normal kidney would have been in good accord with the view that both are excreted by the same mechanism, *i.e.* by glomerular filtration alone, were it not for the doubt cast on his own original interpretation by Höber himself in the above-mentioned paper. For he has recently found that, although cyanol behaves as though excreted only by the glomeruli when the kidney is perfused with a modified Ringer solution, yet when perfused with blood or a hæmoglobin containing solution, the tubule cells appear to be permeable to cyanol.

If this be true also of the mammalian kidney, then the close correspondence of the cyanol and xylose clearances will have to be regarded as a coincidence, although a rather remarkable one. It would then be possible to explain the inhibitory effect of phlorrhizin as due to a cessation of excretion of that fraction of the cyanol excreted by the tubules. But on such a view the remaining glomerular fraction which survives the inhibiting effect of phlorrhizin would be only 40 p.c. of the amount to be expected from the various sugar clearances. This in turn might be attributable to an adsorption of some of the dye in the plasma, rendering it

indiffusible in the same manner as is known to occur with phenol red. With this possibility in mind an attempt was made to determine what fraction of the plasma cyanol was freely diffusible. No appreciable amount of cyanol could, however, be dialyzed out through collodion membranes from plasma taken from rabbits previously injected with the dye either with or without phlorrhizin. This point must therefore be left undecided.

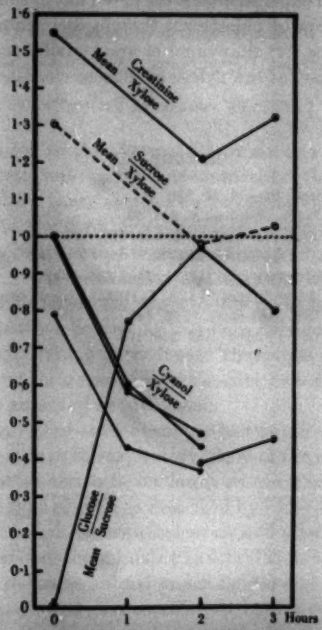

Fig. 1. Effect of phlorrhizin on the cyanol clearance compared with the effect on sugars and creatinine.

But even if Höber's original view that cyanol is a purely glomerular product be correct, then, although the similarity of the cyanol and xylose clearances becomes explicable, the anomalous behaviour of cyanol in the phlorrhizinized kidney still requires some other explanation.

It is clear therefore that further work on the excretion of cyanol is required before these phenomena can be adequately explained.

SUMMARY.

The excretion of the dye "Cyanol extra" has been compared with that of sucrose, xylose and glucose in the rabbit's kidney.

The sucrose clearance has been found to be some 25 p.c. higher than that for cyanol. The xylose clearance was equal to the cyanol clearance within the limits of experimental error in ten out of thirteen comparisons.

Under the action of phlorrhizin the glucose clearance rises to equal the xylose clearance, but the cyanol clearance falls considerably and then averages only 42 p.c. of the xylose or glucose clearance.

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612.211

INFLUENCES WHICH AFFECT THE FORM OF THE RESPIRATORY CYCLE, IN PARTICULAR THAT OF THE EXPIRATORY PHASE.

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In a communication to this *Journal* [Hammouda and Wilson, 1932] the respiratory cycle was described as composed of three phases, the third being the expiratory pause shown as a horizontal line on the graph. We drew attention to the form of the cycle in decerebrate or chloralosed animals, since we believed that it represents correctly the cycle of activity of the central nervous mechanism and that the absence of change in volume of the lungs during the pause proves that the initiation of the inspiratory act could not be due, as had been commonly supposed, to an excitatory stimulus arising in the lungs. The results seemed to show that the respiratory centre governs only the rate and depth of inspiration, expiration being an act of passive recoil.

In a paper published subsequently Sharpey-Schafer [1932] states that there is no pause in the expiratory phase of the normal human cycle, and he holds that an active factor forms an essential part of this phase.

Although the type of cycle as described by Sharpey-Schafer is that most commonly seen, it is nevertheless an undoubted fact that a pause as definite as that seen in animals (Gad [1879] in the conscious rabbit, Head [1889] in the same animal under chloral and ourselves in decerebrate or chloralosed dogs) does occur in the human subject under normal conditions of life. We discussed this question briefly in the appendix to Section I of the paper referred to above. Reference was made to the published records of other authors in which human respiratory cycles are illustrated which show a definite and well-marked expiratory pause. The papers referred to were those of A. Mosso [1878] showing the cycle of a human subject when asleep, Luciani [1911] of a man at rest but awake, and of Haldane and Priestley [1905] in one of their subjects whose breathing was recorded with the body plethysmograph. Later in this paper a figure is shown (Fig. 7) demonstrating this fact. It is evident that

the occurrence of a pause during which the volume of the lungs remains unchanged does not imply anything abnormal in the action of the respiratory centre.

We hope to be able to show that the presence or absence of a pause in the expiratory phase is not related to any active factor apart from the probable reflex control of the frictional resistance of the air passages to the exit of air. An active factor in expiration should be taken to mean a muscular action present in normal breathing and necessary to the discharge of air from the lungs. That such action is not essential is shown by the Drinker respirator. With this apparatus breathing can be maintained at a normal rate in persons with complete paralysis of the respiration, inspiration being effected by the production of a negative pressure external to the body, expiration by passive recoil.

In a paper read recently at a meeting of the Physiological Society, one of us (W. H. W. 1933) has drawn attention to the fact that records of the intra-abdominal pressure do not show any evidence that the abdominal muscles act otherwise in respiration (except in forced expiration) than as a passive elastic membrane, the tension of which is increased by the descent of the diaphragm and which recoils when contraction of the diaphragm ceases. There is, in fact, no indication of any relaxation of tone of the abdominal muscles during normal inspiration such as might be expected to occur if these muscles behaved as the physiological antagonists of the diaphragm. The older view of the part taken by muscular action in expiration was that this was analogous to inspiration and was controlled by an expiratory centre in the medulla. It is now thought that such actions are proprioceptive reflexes originating in the inspiratory muscles. This clearly supports the view that the respiratory centre is essentially inspiratory in function.

A. Fleisch [1928] believes, from his observations on human subjects of the reactions to sudden changes of intra-pulmonary pressure in either direction or to closure of the air inlet during expiration or inspiration respectively, that such proprioceptive reflexes are of importance in normal breathing[1]. Such reactions should continue with diminishing

[1] It may be pointed out that A. Fleisch and other observers have drawn deductions from the results of observations on human subjects using procedures such as the sudden closure of the air outlet or the establishment of pressures resisting expiration which exceed that normally existing in the air passages or can be overcome by the effect of elastic recoil (with normal expansion of the chest about 5 mm. Hg or 6·5 cm. H_2O). The conditions so established can, in fact, only be overcome by muscular action which would not ordinarily be necessary. The reactions to such procedures might very well be regarded as demonstrating the mechanism whereby the breathing is adapted to conditions which may arise but which are abnormal: it is, however, doubtful whether they should be regarded as demonstrating the occurrence of reflex actions taking part in normal breathing.

effect throughout the expiratory phase. No evidence of this can be seen in the cycle of tracheal breathing in decerebrate animals nor, if slight inflation of the lungs be effected during the inspiratory phase, is the termination of inspiration followed by any reversal which is not explained by the release of the passive recoil forces.

Adrian [1933] has found evidence of electrical activity during expiration in the internal intercostals and triangularis sterni of chloralosed cats or rabbits. The activity is of the same kind as that of the externals in inspiration. Adrian informs us that as a rule, however, only the intercostals of the front of the thorax contract regularly, farther back they maintain an even state of contraction or are relaxed. With anæsthetics other than chloralose the expiratory muscles are usually inactive, while in decerebrate animals, in which the effect of an anæsthetic is eliminated, these muscles are sometimes but not always active.

It is evident from Adrian's observations that, under certain conditions, the internal intercostals are active, and it might be expected that when this is so the effect would be shown in the form of the cycle.

Our failure to observe evidence of this on the volume curve may be due to slight differences in the resistance of the air passages due, for example, to differences in the diameter of the tracheal cannulæ employed in our own and in Adrian's experiments.

The fact that the activity observed by Adrian only occurs regularly under certain conditions, and even then does not appear to affect the intercostal muscles throughout suggests that in normal breathing these muscles may, by the maintenance of a constant tone, act as do the abdominal muscles merely as a part of the elastic recoil system.

The main function of the abdominal muscles is to support the weight of the viscera. By their elastic recoil they assist expiration. The intercostal muscles have a similar function in supporting, by their tonic contraction, the intercostal spaces against the negative intra-thoracic pressure. It is possible therefore to regard the internal intercostals as having in normal breathing the same passive rôle as that of the abdominal muscles.

This view seems to be supported by Sharpey-Schafer and Macdonald's observations [1925] on a costo-sternal hoop formed of three ribs, with the nerves and blood vessels intact but with complete separation of their muscular attachments above and below. The thorax is open. When artificial respiration is stopped, rhythmic up-and-down movements (inspiratory and expiratory) of the ribs begin.

They note that while the active contraction of the external intercostal muscles can be observed readily during inspiration no such active con-

traction of the internal intercostals can be observed during expiration, except during the exaggerated movements of dyspnœa. They found, in fact, no evidence that the return of the ribs to their original position in normal breathing was not due to their elastic recoil from the condition of tension produced by the raising of the ribs in inspiration. Thus there does not seem to be any evidence that these muscles undergo relaxation of tonus reciprocal to contraction of the external intercostals, or that they show an increase in their tonic contraction which is effective in assisting expiration.

The apparent difference between the cycle described by Hammouda and Wilson [1932] in chloralosed and decerebrate animals and that usually seen in man, makes it necessary to consider to what this difference is due, and whether it implies a nervous reflex control different from, or additional to, that which they were able to demonstrate under the conditions of their experiments.

In their observations the following factors were absent: (*a*) the frictional resistance of the upper air passages (normally increased at the larynx and nostrils in expiration) to the inflow or outflow of air; (*b*) the possible effect of reflex stimuli arising in the mucosa of that region; (*c*) the effect of influences arising in the nervous system above the mid-brain (reflex or cortical) which may be necessary in the adaptation of the breathing to changes of posture or active bodily movements.

The observations recorded below have been designed to elucidate these questions, by determining (*a*) the form of the cycle in the same animal breathing through the normal channels (glottis, etc.) as compared with breathing through a tracheal cannula; (*b*) the effect of a resistance to the outlet or inlet of air; (*c*) the effect of an anæsthetic, and (*d*) the effect on the cycle or rhythm of breathing of any reflexes which might arise in the upper air passages.

I. Comparison of the cycle in tracheal and mask breathing.

Method. Fig. 1 shows the apparatus devised for this purpose. Chloralosed or decerebrate dogs were used. The trachea was opened sufficiently and the two-way tracheal end of the cannula (*B*, Fig. 1) tied in. It will be seen that air can therefore pass directly to the upper air passages or, if the mask exit is blocked and the tracheal open, directly to the tracheal. The rubber face-piece was modelled to fit tightly to the muzzle of a dog of the size usually employed. The face-plate *b* and

attached face-piece is first fixed in position by two tapes, not shown in the diagram, attached to the sides of the mask and tied behind the head of the animal. In actual use the nose of the dog is much closer to the opening in the plate *b* than is shown, the dead space being then from 20 to 30 c.c. The opening in the plates *a* and *b* is large enough to prevent

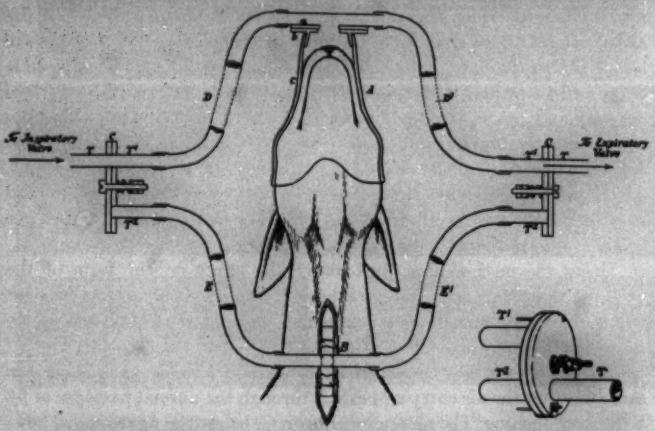


Fig. 1. Apparatus for alternative tracheal and mask breathing. *A*, mask; *a*, detachable brass plate, carrying inlet and exit tubes, which can be clipped on to *b* to a flange on which the conical rubber face-piece *c* is attached; *D*, *D'*, inspiratory and expiratory rubber connections from mask to air switches; *B*, two-way cannula with *T* connection to *E*, *E'*, inspiratory and expiratory rubber connection from trachea to air switches; *C*, *C*, air switches *in situ*. Inset, same shown in perspective. *T*, *T*, connection to inspiratory and expiratory valves; *T'*, connection to mask; T^2, connection to trachea. In the diagram the switches are shown in position for mask breathing; in the drawing inset they are shown in position for tracheal. A catch is seen on the outer plate of the switch and two stops on the inner plate. The outer plate can be rotated and is arrested by one stop opposite T^2 by the other opposite *T'*. The tubes and connections are all of ½ in. diameter.

pressure on the nostrils or their surroundings. The plate *b* also carries a short metal tongue which is passed between the incisor teeth partly to steady the mask, partly to keep the jaws slightly open. Between the plates *a* and *b* is a rubber washer which makes the joint airtight.

The tubes *E* and *E'* from the tracheal cannula, and the plate *a* of the mask, are attached when the preparations for the experiment are com-

plete. For observations where no great changes of pressure occurred the simple rubber face-piece gave quite satisfactory volume records.

In the conduct of an experiment the air tubes T^1, T^2 of the air switches are held firmly in clamps placed on either side of the animal. To switch over from the mask or *vice versa* (Fig. 2), the observer, holding the tube T of each switch, one in the right the other in the left hand, rotates them rapidly in the correct direction at any desired moment in the cycle (by observation of the kymograph record or signal from a colleague). It will be seen from the figures that this can be done without any appreciable delay. Observations with this method have been made on the effects of inflation and deflation of the lungs with tracheal and mask breathing, the tubes from the trachea and mask respectively being passed through the cover of the chamber described by Wilson and Hammouda [1928]. The results were identical whether the breathing was by the normal channels (mask) or by the tracheal outlet: indicating that the passage of air in either direction over the mucosa has no effect.

Using the method described above the effect on the form of the cycle of switching over from tracheal breathing to breathing through the normal channels has been examined. Examples of the results are shown in the following figure (Fig. 2).

From Fig. 2 it will be seen that the general form of the cycle is the same whether the air entry and exit is through the normal passages or by a tracheal opening. The measurements given below the figure show, however, certain quite distinct differences in the phases. The duration of the whole cycle (rate of breathing) is little affected. In tracheal as compared to mask breathing the depth of inspiration is greater, its duration in some cases slightly less. The first phase of expiration (b, c) is slightly shortened, the expiratory pause is of longer duration. The line of expiration (phase 1) is less oblique and less curved.

It will be shown later that these differences are explained by a slightly greater frictional resistance to the air passing through the upper air passages in both inspiration and expiration. The resistance offered by the upper air passages is evidently very small in animals under the conditions described, and in these observations appears to be greater in the inspiratory than the expiratory phase. It is evident from Fig. 2 B that the cycle after vagotomy is the same whether the breathing takes the ordinary channels or is through the tracheal outlet; the only difference between the two curves (M and T) being a slightly greater obliquity and rounding of the first phase of expiration in the mask breathing as compared to the tracheal.

In Fig. 2 C a similar observation is shown made on a dog under chloralose, the breathing being switched over from tracheal to mask. The whole cycle was slightly shorter and the depth of inspiration was greater in proportion to the duration than in Fig. 2 A.

Fig. 2. Comparison of cycle in tracheal and mask breathing. **A.** Dog. 5 kg. Morphine, 0·1 c.c. of 5 p.c. sol. per kg. C. and E. Decerebration immediately in front of ant. colliculi 3 hours before time of observation. A before, B after, section of cervical vagi. *M*, breathing through normal passages (mask); *T*, through tracheal cannula; arrow shows moment of change-over from *M* to *T*. Means of cycles before and after switching over. Rate of breathing: 19·5 per min. in both *M* and *T*. Duration of inspiration: *M*, 0·75; *T*, 0·67 sec. Duration of expiration: 1st phase, *M*, 0·9, *T*, 0·8 sec.; pause, 1·46; 1·64 sec. *a*, *b*, inspiration; *b*, *c*, expiration, 1st phase; *c*, *d*, pause; *a'*, *b'*, *c'*, *d'*, same for tracheal breathing. **B.** After section of vagi in neck. Rate of breathing: 7·7 per min. **C.** Dog. 5 kg. Morphine and C. and E. as above. Chloralose 0·07 g. per kg. *T*, tracheal breathing; *M*, mask breathing; arrow shows moment of change-over. Rate of breathing: *T*, 21; *M*, 19½ per min. Duration of expiration: *T*, 2·05; *M*, 2·3 sec. Duration of inspiration the same in *T* and *M*. **D.** Dog. Chloralose. Abnormal type of cycle resembling that sometimes described as normal for man. Rate of breathing: *T*, 21; *M*, 20 per min. Duration of expiration: *T*, 1·74; *M*, 1·87 sec. In all records read left to right; inspiration down, expiration up.

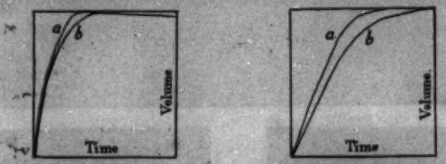
In this case, although the duration of inspiration was little affected by the change, the depth was definitely diminished in mask breathing as compared to tracheal. The expiratory phase shows a similar but more marked change than in Fig. 2 A. The pause being almost abolished.

In Fig. 3 the expiratory phase of typical examples of cycles of mask and tracheal breathing respectively in the two cases have been brought to the same duration and volume scale.

In the curves in Fig. 3, *a* is that of the expiratory cycle in tracheal breathing, *b* that in breathing through the normal channels.

The curves may be compared with those given later, in which an artificial resistance has been introduced in the exit of air with tracheal breathing.

The observation recorded in Fig. 2 D is from a chloralosed dog. The cycle closely resembles in type that described by Schafer [1932] as being a common normal type in man (*vide* that author's Fig. 2). We have



(1) Graph A, Fig. 2. (2) Graph C, Fig. 2.

Fig. 3. Form of the expiratory phase in tracheal and mask breathing. (1) Expiratory phase of cycles in Fig. 2 A. *a*, tracheal breathing; *b*, mask breathing. (2) The same for two cycles of Fig. 2 C. (1) is from a decerebrate, (2) from a chloralosed animal. The depth and duration have been brought to the same level in each case.

occasionally, though rarely, met with this form in animals under chloralose, but in our experience it is never seen after decerebration. It will be observed that there is no change in the form of the cycle on switching over from tracheal to mask breathing. There is no significant alteration either in the depth of inspiration or the duration of either phase. It is evident that the absence of the pause, and the regular and continuous rise in the line of expiration, are in no way connected with the effects of frictional resistance or reflex influences arising in the upper air passages, and it seems unlikely that the resistance of the bronchi could explain the form. The fact that the line of expiration closely resembles that of inspiration suggests that an active element exists in expiration in this type of cycle which is absent in that commonly seen. This type of cycle is the same as that described by Gad as occurring in the conscious rabbit breathing through a mask, before the animal has become quiescent after the first emotional disturbance. It also resembles the cycle of panting (except in rate) which one of us [Hammouda, 1933] has shown to be

related to the action of a centre situated in the thalamic region. In the emotional type of cycle described by Gad and in panting both expiration and inspiration are active processes.

That the subject whose respiratory cycle is recorded in Schafer's figure referred to above was conscious of his breathing and that the rhythm therefore cannot be regarded as normal is suggested by the varying depth of inspiration and particularly by the irregularity in the point reached in expiration, the base line of expiration being normally a straight line unless affected by postural changes.

II. Effect of resistance to the entry or exit of air on the form of the expiratory and inspiratory phases respectively.

From the observations recorded in Fig. 2 A, C, it is evident that certain changes take place in the duration and form of the phases which may probably be explained by a greater frictional resistance to the passage of air through the upper air passages as compared to the tracheal outlet. The following observations are designed to show the effect of resistances of known amount introduced into the expiratory or inspiratory air way to a tracheal cannula.

Method. A cannula is tied into the trachea and to this is attached a T tube, one branch of which goes to the expiratory the other to an inspiratory valve. Between the cannula and the valves a connection is made with a mercurial manometer which thus registers the resistance against which the animal is inspiring or expiring. The valves are connected in the usual way with a 10-litre bottle and a bellows volume recorder. The rubber tube connecting either the inlet or exit valve with the air reservoir (whichever phase it is desired to study) is passed under a clamp, by depressing the handle of which the tube is compressed. An adjustable stop is provided by which the descent of the clamp can be arrested at any point. The passage of air can thus be obstructed to any desired extent. The exit or inlet tube having been placed in position, a few normal cycles are recorded, and the clamp is then closed at any suitable point in the cycle.

1. *Effects of resistance to inspiration.*

In Fig. 4 the effects of four different degrees of resistance are shown before and after section of the vagi. The curves in the different cases have been analysed and are represented in a semi-diagrammatic form in the two graphs (III, IV) below the records.

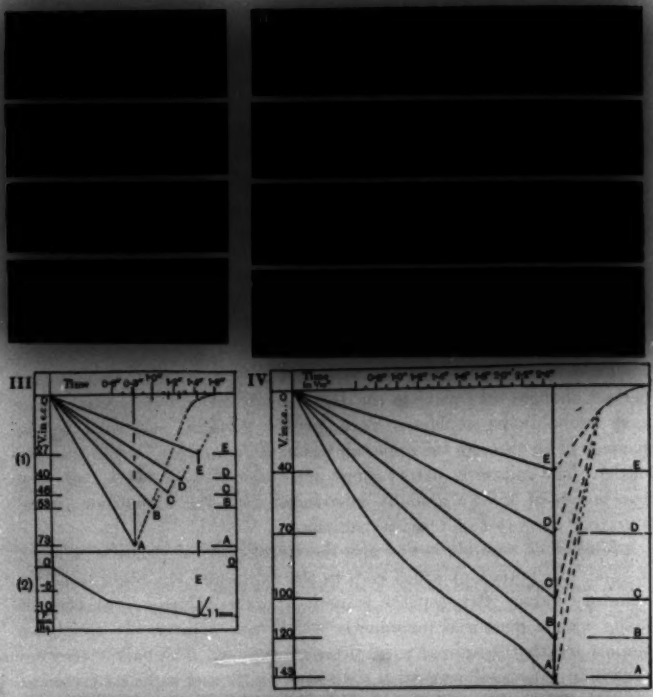


Fig. 4. Effect of resistance to inspiration on the cycle. Dog. 5 kg. Morphine, C. and E. and chloralose. I. Vagi intact. Resistance: Graph (1) – 4 mm.; (2) – 6 mm.; (3) – 8 mm.; (4) – 11. II. Vagi cut. Resistance: Graph (1) – 4 mm.; (2) – 6 mm.; (3) – 12 mm.; (4) – 15 mm. In all graphs read left to right; inspiration down, expiration up. Downward arrow shows point at which tube to inspiratory valve was clamped. Upward arrow shows release of clamp. The cycles affected are shown on the manometer record by fall of pressure. Upper tracing, volume record; lower, Hg manometer. III. Semi-diagrammatic representation of the inspiratory phase in the four cases before vagotomy. IV. The same after vagotomy. In III, graph (2) represents the pressure change during inspiration in graph I (4).

Vagi intact (I, III)

	A.	B.	C.	D.	E.
P	0	– 4	– 6	– 8	– 11
T	0·8	1	1·15	1·25	1·45
V	73	53	46	40	27

Vagi cut (II, IV)

	A.	B.	C.	D.	E.
P	0	– 4	– 6	– 12	– 15
T	2·45	2·45	2·45	2·45	2·45
V	143	120	100	70	40

P = resistance to inspiration in mm. Hg; *T* = duration of inspiration in sec.; *V* = depth of inspiration in c.c.

The manometer record of E only is shown in graph III, the pressure changes in the other cases being similar but of lesser extent.

An inspection of the results illustrated in Fig. 4 shows the following effects of resistance to inspiration in animals under the conditions of the experiments: (*a*) the rate of breathing (total duration of cycle) is unaffected; (*b*) the depth of inspiration is diminished in proportion to the resistance; (*c*) the duration of inspiration is increased; (*d*) the pressure curve (with constant resistance) shows at first a rapid, followed by a slower but continuous increase in negative pressure indicating a gradual increase in the force of the inspiratory muscular contraction which, in view of the progressively increasing resistance to expansion in inspiration under all circumstances, evidently represents the normal course of action of these muscles.

After section of the vagi (IV) the results are the same with one exception, namely that the duration of the inspiratory phase is identical in all four cases, which would indeed be expected if it be remembered that the duration of this phase after vagotomy represents the duration of the discharge from the respiratory centre unaffected by reflex influences reaching it from the lungs.

A further point of interest appears from a comparison of the relative effect of resistance to the inspiratory phase before and after vagotomy. Hammouda and Wilson [1932] drew attention to the fact that the depth reached in a given time in the inspiratory phase after vagotomy is the same as in the normal cycle before vagotomy, although in the former owing to the absence of the inhibitory reflex excited by expansion the discharge from the centre (and inspiration) continues for the full period. This observation is confirmed by the results shown in Fig. 4. If curves A, B and C be compared in graphs III and IV of that figure, A being the inspiratory phase without resistance, B in the two cases with – 4 and C with – 6 mm. respectively, the depth reached in the same lapse of time, namely 0·8, 1·0 and 1·15 sec., is almost exactly equal.

These results support the view we expressed that, while the whole central mechanism is under the influence of the chemical stimulus, the rate of the rhythm and the duration of the discharge are controlled by stimuli arising in the lungs, but the force of the discharge and so the strength of the contraction of the inspiratory muscles is not under this reflex control but is dependent on the state of chemical excitability of the centre.

2. *The effect of resistance to expiration.*

In the observations illustrated in Fig. 5 the effect of three different degrees of obstruction to the outlet is shown (I (1), (2), (3)). In

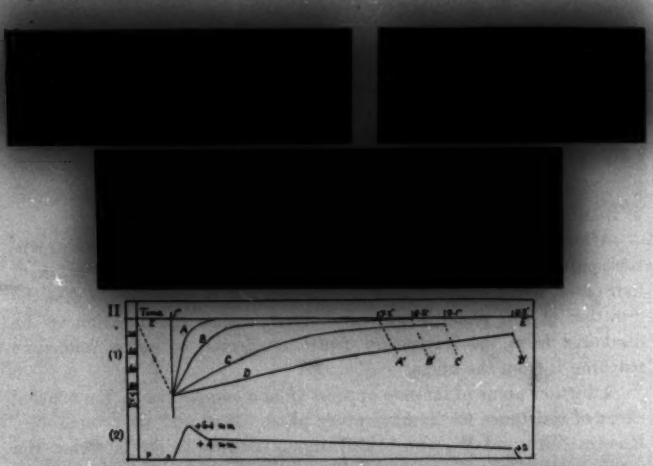


Fig. 5. Effect on the cycle of resistance to expiration. Dog. 4 kg. (same animal as in Fig. 6) after chloralose, 0·07 g. per kg. I. Record of results. Initial resistance: Graph (1), 2·5 mm. Hg; (2) 4 mm. Hg; (3) 6·4 mm. Hg. *a*, volume record; *b*, Hg manometer record. Downward arrow shows point at which rubber connection to expiratory valve was compressed. Upward arrow shows release of clamp. II. Semi-diagrammatic representation of cycles in the three cases. Broken line - - - - - - - -, inspiration; full line ————, expiration. *E*, *E*, base line of expiration; *P*, base line of manometric pressure. *A*, normal expiratory phase without resistance (graph 1); *B*, expiratory phase with resistance = 2·5 mm. (graph 2); *C*, expiratory phase with resistance = 4 mm.; *D*, expiratory phase with resistance = 6·4 mm. *A'*, *B'*, *C'*, *D'* = termination of expiratory phase. The curves have been plotted from tracings and measurements. The duration of the normal cycles in the three cases was not identical for comparison; the duration of the cycles shown diagrammatically has been reduced to the level of the normal cycle in graph (2). Duration of inspiration: 1 sec.; depth, 83 c.c. Duration of expiratory phase: *A* 6·2 sec., *B* 7·2 sec., *C* 8·1 sec., *D* 10·2 sec. Sustained inflation in excess of normal in *B* 0 c.c., *C* 8 c.c., *D* 20 c.c., measured from the base line of expiration *E*, *E*.

graph II (1), (2), the expiratory phase of the records is presented in a semi-diagrammatic form. The changes in the expiratory phase due to the resistance to the issue of air are well seen in graph II in which the normal

cycle and the three subjected to resistance are compared. The changes are as follows: (*a*) the duration of the whole cycle is increased (slower rhythm) owing to the increased length of the expiratory phase; (*b*) the pause is abolished, being replaced by a gradually rising line the curvature of which diminishes as the resistance increases; (*c*) the base line of expiration is lowered in the direction of inflation.

The results confirm our previous observation that a lowering of the base line of expiration [Hammouda and Wilson, 1932, Figs. 6, 7] by a slight resistance, external to the thorax, resisting collapse, is accompanied by a slowing of the rhythm depending on the degree to which collapse is prevented. The fact that no change in the form of the expiratory phase, the pause remaining, is to be seen in those records is due to the air outlet being unobstructed (the resistance was external to the thorax).

The explanation of the slowing is evidently that the sustained slight relative inflation by its tonic inhibitory effect diminishes the rate of discharge from that part of the central mechanism controlling the rhythm.

The intra-pulmonary pressure curve (Fig. 5 II (2)) is at its highest at the commencement when the forces of elastic recoil are at their maximum, the pressure gradually falling to the base line or until the next inspiration begins, a result which would be expected if the discharge of air were due simply to passive elastic contraction. There is no evidence here of an active muscular element in expiration such as is seen in the pressure curve of inspiration shown in Fig. 4 III (2). The form of the expiratory phase resulting from resistance to the outlet will be compared with this phase in certain human and other respiratory cycles.

Davies, Haldane and Priestley [1919] described the effects on breathing in man of resistance to inspiration and expiration (an obstruction being placed in both inlet and outlet). Owing to the type of spirometer used in their experiments, in which expiration is not recorded, the form of this phase of the cycle is not shown and it is impossible to say whether or not the base line of expiration is lowered by sustained inflation. They demonstrate a well-marked slowing of the rhythm due mainly to increase in the duration of expiration, with some increase in the duration of inspiration. This is in agreement with the observations recorded in this paper. Our observations, however, show both in anæsthetized and decerebrate animals, in which the effect of an anæsthetic is eliminated and the automatic centres may therefore be regarded as showing their normal reactions, an immediate decrease in depth of inspiration proportionate to the increase of resistance. Davies, Haldane

and Priestley find, on the other hand, an immediate and very marked increase in the depth of breathing, for example, in their Fig. 4 in the cycle immediately following introduction of a considerable resistance inspiration is doubled in depth, which they ascribe to an increase in the chemical stimulus. With a similar pressure in our experiments on animals, in which the influence of centres above the level of the mid-brain is absent, the depth would have been decreased by more than half.

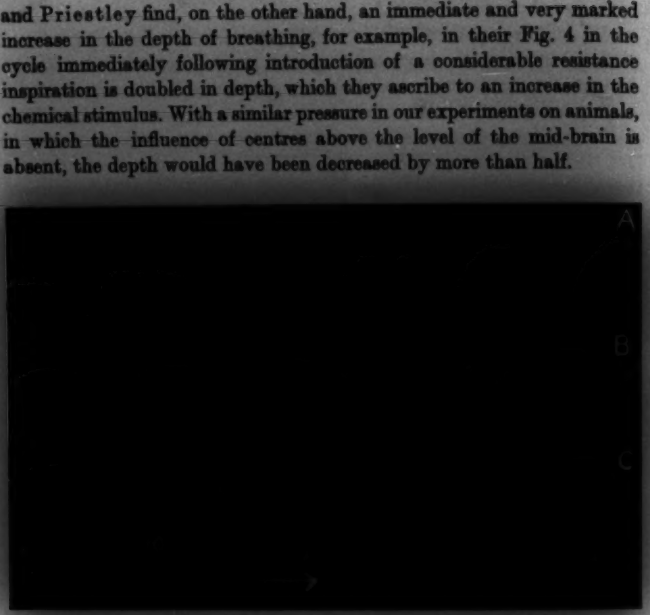


Fig. 6. Effect of anæsthesia on the cycle. Dog. 4 kg. Morphine, C. and E. No operative interference. Breathing through mask. Inspiratory and expiratory valves to 10-litre air reservoir. Bellows volume record. A. Fully under anæsthetic. B. Light. C. Effect of anæsthetic passing off. Rate of breathing: A 14, B 13·7, C 13 per min. Duration of cycle: A 4·3 sec., B 4·4 sec., C 4·7 sec. Duration of inspiration: A 1·15 sec., B 1·15 sec., C 1·15 sec. Duration of expiration: A 3·15 sec., B 3·25 sec., C 3·55 sec.

3. *Effect of anæsthetics on the cycle.*

In view of the fact that in man during sleep the expiratory phase of the cycle has been described as showing the pause, it appeared to be of interest to record the cycle of a dog breathing through a mask during recovery from anæsthesia. The anæsthetic used was C.E. No operative procedure had been carried out.

Fig. 6 shows the form of the cycle in a dog under different degrees of anæsthesia. In C when the animal was recovering from the effect of the anæsthetic but was still quiescent, the form of the cycle resembles closely

that commonly seen in man. The pause has disappeared and the record of the expiratory phase shows a characteristically curved line which will be shown later to correspond to an increased resistance to the outlet. The slight slowing of the rhythm is dependent on the lengthening of this phase and is evidently the reflex effect of the more sustained inflation. That there is no increase in the resistance to inspiration is suggested by the fact that the duration of this phase is the same in the three cases, and that the depth is not diminished but slightly increased in C.

Fig. 7. Human respiratory cycles. **A.** Subject G.V.A. Stethograph record. Rate of breathing: 8·5 per min. Duration of inspiration: 1·5 sec. Duration of expiration: 5·5 sec. Ratio inspiration/expiration 1 : 3·6. **B.** Subject M.A.K. Stethograph. Thorax. Rate of breathing: 6 per min. Duration of inspiration: 2·8 sec. Duration of expiration: 7·2 sec. Ratio 1 : 3. **C.** Same subject. Stethograph. Thorax. Rate of breathing: 6 per min. Ratio 1 : 3. **D.** Same subject. Abdominal record. Stethograph. Rate of breathing: 4 per min. Ratio 1 : 3. Both subjects were adult men: awake, in an easy sitting posture. Read right to left in all curves; inspiration down, expiration up.

4. *Examples of human cycles.*

Fig. 7 shows stethograph records from two adult male subjects, awake but quiescent. In A, B and C the stethograph was on the thorax. D is from the same subject as B and C, the record being in this case of the abdominal movements.

The breathing is slow in both subjects, in particular in K. on the occasion on which record C was taken. The cycle in A shows an expiratory

form not unusual in man (compare *l.c.* Schafer, Fig. 1). In both B, C and D a definite pause is seen, especially pronounced in C and D.

In view of the apparent difference in the form of the expiratory phase of human and other cycles in which the depth and rate of breathing vary, it appeared to be of interest to compare the expiratory phases in certain of the cycles illustrated or referred to in this paper by reducing them all to the same level of volume and duration and plotting the results in the form of curves.

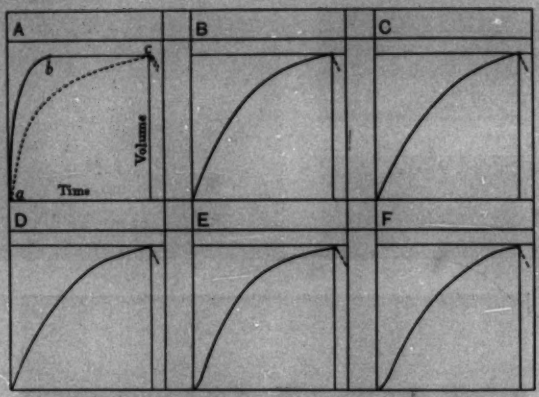


Fig. 8. Semi-diagrammatic representation of the expiratory phase of various cycles. **A.** Normal cycle. (Dog under chloralose, Fig. 5.) *a*, *b*, 1st phase of expiration; *b*, *c*, pause. Dotted line shows the form of curve *a*, *b* if extended for duration *a*, *c*. Rate of breathing: 8½ per min. **B.** Cycle from same figure (5) with resistance to expiration of 2·5 mm. Hg. Rate of breathing: 6½ per min. **C.** Cycle from Fig. 6 C. This is from the same animal as A and B before injection of chloralose or any operative procedure. The dog was recovering from C. and E. anæsthesia. Rate of breathing: 13 per min. **D.** Human cycle (Fig. 7 A). Rate of breathing: 8½ per min. **E.** Human cycle (from Schafer's Fig. 1, 2nd cycle, 1932). Rate of breathing: 9 per min. **F.** Human cycle (from Barcroft and Margaria, Fig. 1, VI, 1931). Rate of breathing: 21 per min.

5. *Comparison of the expiratory phase of various cycles.*

This has been done and the resulting curves are shown in Fig. 8, A, B, C and D being from examples of respiratory cycles figured by us; E and F from cycles described by other observers.

Graph A represents the expiratory phase seen in Fig. 5 without any obstruction to the outlet with tracheal breathing; the only frictional

resistance to the exit of air from the lungs being that of the bronchioles and bronchi. The short first phase *a–b* and the prolonged pause *b–c* may be compared with that seen in the example we have given elsewhere [Hammouda and Wilson, 1932, Fig. 1, p. 84] of the normal cycle in the anæsthetized or decerebrate dog. The dotted line represents the portion *a–b*, extended to the same duration as in the other diagrams. It represents the progress of the discharge of air from the lungs by the various forces which bring this about.

Graph B represents the expiratory phase in a cycle from the same record as A but with a slight added obstruction to the tracheal outlet (Fig. 4 II, and graph C). It seems clear from a consideration of these curves that whether or not a pause occurs is dependent solely on the freedom or the reverse of the exit of air. This particular example is selected as the added resistance which abolished the pause and gave rise to this curve was sufficient to produce an initial effective pressure of 2·5 mm. Hg, which is about the same as the expiratory pressure in the human air passages in quiet breathing, namely 2–3 mm. [Starling, 1926].

Graph C is the expiratory curve from one of the cycles of a dog recovering from anæsthesia shown in Fig. 6 C. This was the same animal as A–B before administration of chloralose. The expiratory phase in A of Fig. 6 shows a well-marked pause, and if treated as the cycle in C has been would give a curve exactly similar to that shown in graph A of Fig. 8. This suggests that as consciousness is recovered the normal resistance of the upper air passages, due largely to the narrowing of the glottis in expiration, returns. This probably also explains the fact that, while in sleep in the human subject a pause in the expiratory phase is seen, this is not usually the case in the waking state.

Graph D is from the record A shown in Fig. 7, the breathing of a human subject sitting in a restful attitude.

Graph E represents the expiratory phase of a respiratory cycle of the subject whose breathing is recorded by Schafer [1932, Fig. 1].

Graph F represents similarly the expiratory phase of one of the cycles (2) in record VI of Fig. 1 of the paper by Barcroft and Margaria [1931] referred to above. This example is selected partly as it is a record of the volume expired into a spirometer and not a stethograph record of the thoracic movements as are D and E, and partly because the rate of breathing was considerably quicker (excess CO_2 in air), namely 21 per min., than in D or E, the other two human cycles, in which the rates were respectively about 8 in D and 9 in E.

The close resemblance of all these curves, in particular B and C (from the same animal) and D, suggests that the controlling factor upon which the shape of the curves and the absence of a pause depend is the same as in B, namely a slight peripheral resistance to the exit of air; as pointed out above this need not exceed the normal human expiratory pressure.

The form of the curves, and the fact as pointed out in discussing Fig. 4, that during expiration against a resistance the intra-tracheal pressure falls gradually from the commencement until the end of expiration, is entirely in keeping with what would be expected if the force by which air is expelled from the lungs were the recoil of an elastic system beginning at a maximum and progressively diminishing.

The observations do not give any support to the view that normal expiration is in part brought about by a muscular contraction specifically directed towards that end, in any way comparable to the active function of inspiration.

III. Stimuli arising in the upper air passages.

It seemed possible that the form of the cycle might be influenced by reflexes arising in the glottis or other parts of the upper air passages from the movement of air over the surface or other stimuli. We propose to give here a brief account of some observations we have made on this subject.

Method. The trachea (dog) was opened low down in the neck and a bent glass cannula was tied in. This was connected in the usual way with valves and other apparatus for obtaining a volume record of the breathing. Into the upper end of the trachea a similar cannula was tied at about 2 cm. from the glottis. It will be seen that the upper air passages and the lungs are entirely disconnected, and therefore that any changes in the breathing which may accompany alterations in the condition of the upper air passages must be of reflex origin. A mask was in some observations attached, in others cannulæ were introduced into the nostrils. A current of air or other gas can be blown through from the upper tracheal cannula producing an air current in the direction of expiration, or by sucking air from the cannula in the direction of inspiration.

No change in the form of the cycle was observed as the result of any of the procedures tried, with the exception of a decrease in depth of inspiration which will be referred to below. Currents, whether of air, oxygen or CO_2, in either direction produced no change in the breathing provided there was no change of pressure.

Marked slowing of the rhythm accompanied by a decrease in depth of inspiration were, however, observed accompanying pressure changes in the air passing through the upper air passages.

An example of this is shown in Fig. 9.

It will be observed that a slight positive pressure (4 mm. Hg) affecting about 2 cm. of the upper end of the trachea, the glottis and the parts

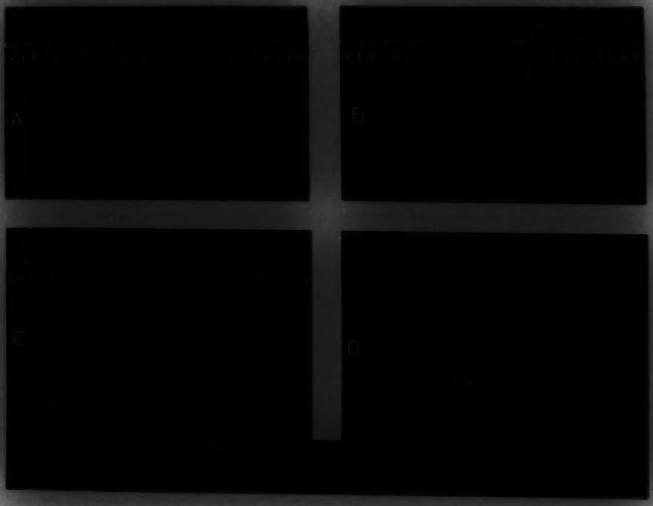


Fig. 9. Effect of pressure changes in the upper air passages. Dog. 5 kg. Decerebrate (after morphia and C. and E.). **A.** Positive pressure of 4 mm. Normal rate, 15·3; with pressure, 12 per min. Mean increase in duration of cycle with pressure, 1·15 sec. (32 p.c. increase). **B.** Positive pressure of 10 mm. **C.** Negative pressure of − 14 mm. Normal rate, 18; with pressure, 16 per min. Increased duration of cycle, 0·4 sec. (11·8 p.c.). **D.** Pressure of 4 mm. Hg after vagotomy. All curves read left to right. Upper line, volume record; lower line, manometer. Inspiration, down.

above it is sufficient to produce a very marked slowing of the rhythm, the depth of inspiration being decreased from about 60 to 45 c.c. without, however, any significant increase in duration, the slowing being due to the increase in the duration of the expiratory pause. With a higher pressure (graph B) a complete inhibition of the rhythm is produced which continues for 8 sec. after the pressure has been released. Graph C shows

the effect of a negative pressure. It will be seen that while a definite slowing is produced, the effect, in view of the higher pressure, is much less marked than with a positive pressure.

It will be observed from graph D that a small positive pressure inhibits the rhythm after section of the vagi and that as in A before section this continues for some seconds after the pressure has fallen.

Attention is drawn to the fact that the pressure stimulus required to produce cessation of the rhythm is considerably less than that required before section, this may be attributed to the fact that section of the vagi lowers the excitability of the centre owing to the absence of the tonic impulses from the lungs which normally augment the responsiveness of that part of the centre controlling the rhythm. If our view be correct it is evident that a smaller stimulus will be required to inhibit the activity of this part of the centre after than before section of the vagi.

The application of a strong solution of cocaine to the upper end of the trachea, the glottis and the mucous membrane immediately above it abolishes these phenomena; it seems therefore probable that the effects are due to the stimulation of the sensory endings of the superior laryngeal nerves in the mucosa or the immediately subjacent tissues. That this is so is probable if it be remembered that Rosenthal [1862] showed that slowing or cessation of the breathing was the constant effect of exciting this nerve. We have not yet obtained experimental evidence as to whether any reflex effects on the breathing occur arising in the upper air passages after section of the superior laryngeal or glossopharyngeal nerves. We cannot therefore at present state precisely the area from which these stimuli arise.

The reflex slowing described as the result of pressure change in the upper air passages may in part account for the very marked decrease in the rate of breathing recorded by Haldane and his co-workers, referred to above, in the human subject; it cannot, however, explain the slowing obtained from resistance to expiration illustrated in Fig. 5 of this paper, but may account for the slowing seen in some cases of mask breathing in the dog, and that seen in Fig. 6.

The similarity of the phenomena described above to the effects of expansion of the lungs suggests that they form a part of the same reflex function, and that while the origin of the former is probably in the terminations of the superior laryngeal in the mucosa of the larynx and upper third of the trachea, that of the latter may be in the terminations of the pulmonary vagi in the mucosa of the bronchi and bronchioles.

No stimuli to the upper air passages which we have yet employed have produced the characteristic acceleration of the rhythm which accompanies deflation of the lungs.

Discussion.

Our former investigations on the effects of inflation and deflation of the lungs demonstrated that two influences reached the centre from the lungs; the one inhibitory of inspiration, the other augmentor of the rate of the rhythm. In that investigation no evidence was found of any reflex controlling expiration or the form of that phase of the cycle, with the exception of the slowly developing increase in the tonus of the expiratory muscles resulting from sustained and considerable expansion of the lungs, which might even develop into a reversal of the normal process, expiration becoming the active, inspiration the passive phase. The reflex production of an increased expiratory tonus has no counterpart in the normal cycle even in the slow rhythm of slight inflation. It is shown after vagotomy, and appears to correspond to the reflex effects described by **Fleisch** which he regards as evidence of the proprioceptive reflexes arising in the muscles of inspiration and reacting on the expiratory phase. That proprioceptive reflex effects arise as in other skeletal muscles is probable. As pointed out in the introduction to this paper the abdominal muscles do not show any evidence of their presence, and the form of the expiratory phase with tracheal breathing in the decerebrate animal, even in hyperpnœa, indicates either that these reflexes have little influence on the expulsion of air from the lungs or that the changes of tone in the internal intercostals are counterbalanced by corresponding changes in the opposite direction in the external intercostals.

The observations recorded in this paper appear to explain the difference in the form of the expiratory cycle observed in dogs with tracheal breathing, and sometimes in man, and the common human type.

It is, however, evident that something more is required to explain the fact that with very varying rate of breathing in the conscious human subject and probably in animals in ordinary conditions of active life, the diminishing volume of the lungs reaches the base line of expiration immediately before inspiration begins without any intervening pause.

This is well seen in **Barcroft and Margaria's** figures [1931] of breathing accelerated by either excess of CO_2 or by effort. In the decerebrate dog the only marked change under similar conditions (CO_2) is a progressive shortening of the pause, the duration of the inspiratory phase and the first phase of expiration undergoing little change whatever

the depth. In Margaria and Barcroft's observations, while there is slight diminution in the duration of inspiration, the shortening of the cycle with increased rate is mainly at the expense of the expiratory phase.

Our observations provide no evidence that the rate of discharge of air from the lungs is regulated by any action of the expiratory muscles. Were this so its effect should be seen in the cycle of breathing in decerebrate animals in which such reflexes as may occur would not be suppressed by the action of anæsthetics. The evidence is, in fact, entirely in favour of the control of expiration being effected by a mechanism regulating the frictional resistance at the exit. The increase in the dead space in dyspnœic conditions suggests that there is some reflex relaxation of the bronchioles in rapid or deep breathing in addition to the automatic dilatation of the air passages facilitating both ingress and egress of air which accompanies any increase in the volume of the lungs. The slight change in the duration of either the inspiratory or expiratory phase which occurs in dyspnœa in the tracheal cycle of decerebrate animals excludes the possibility of the regulating mechanism acting within the lungs. The regulation of the rate of discharge of air must therefore be through the accessory muscles of respiration in the upper air passages. The exact adjustment of the aperture of the glottis and the external nares to the depth and rate of inspiration is well known, in some animals such as the horse this reflex is essential to the proper adjustment of respiration in muscular exertion; even in man, where mouth breathing is normal under such conditions, it is of some importance.

The usual form of the expiratory phase of the respiratory cycle in man implies that this reflex control extends also to the aperture of the outlet during expiration, the diminished frictional resistance with deep breathing cannot be regarded as merely the passive effect of the expansion during inspiration. The extent of this regulation of the exit and how it is effected is not so evident.

Hess [1930], from observations of the position of the dome of the diaphragm with the open thorax, has observed changes in the tone of that muscle increasing progressively with decreasing volume of the lungs. He regards these effects of collapse of the lungs and thoracic walls as regulatory of the process of expiration. He states also that reflex effects similar to those produced by inflation and deflation of the lungs can be elicited by passive movements of the thoracic walls without change in the lung volume, suggesting that the nervous control of the breathing is not entirely dependent on impulses received by the centre through the pulmonary vagi. Creed and Hertz [1933] have recently produced evi-

dence which they believe confirms the results of Hering and Breuer and Head, using the latter author's method of the diaphragmatic slip, in rabbits, that contraction of the diaphragm is the normal response to deflation. We have never observed any indication of such a reaction to deflation of the lungs on the volume record with the intact thorax. The conditions, which we are at present investigating, under which such tonic contractions of the diaphragm occur appear to be rather complex. A study of Creed and Hertz's figures indicates, as was originally pointed out by Schenk [1903], that this response to deflation does not occur unless the collapse of the lungs is sudden and complete; there does not seem to be, as suggested by Hess, an increase of tone increasing progressively with the diminution in the lung volume.

The regulation of the frictional resistance to the outflow of air through the glottis and external nares might be effected either by a control of the rate of relaxation of the abductor muscles (and dilator of nares) or of the contraction of the adductors. The sustained dilatation of the nares in hyperpnœa, in animals in which this function is important, suggests that the former is the more probable. This reflex control of the resistance in the upper air passages to the inlet and outlet of air seems to be absent in anæsthetized and decerebrate animals, suggesting that it is related to the activity of centres above the level of the mid-brain.

We hope by a modification of the method described for examining the reflex influence of stimuli arising in the upper air passages to further investigate this question.

Summary.

1. The presence of the upper air passages in anæsthetized or decerebrate animals diminishes the depth of inspiration and prolongs the first phase of expiration, as compared to tracheal breathing.

2. Resistance to inspiration is shown to cause a decrease in depth and an increase in the duration of the inspiratory phase of the cycle.

3. The expiratory pause is shown to occur in the normal human cycle under certain conditions.

4. Resistance to expiration about equal to that normally seen in the air passages during that act abolishes the expiratory pause, the presence or absence of which is therefore dependent on the extent of the frictional resistance to the exit of air offered by the upper air passages.

5. The form of the expiratory phase of the respiratory cycle usually seen in man is shown, by comparison with the effects of air resistance to the air exit in tracheal breathing in animals, to be due to the resistance

to the exit of air through the upper air passages which must vary with the depth and rate of breathing and is, it is suggested, under some reflex nervous control.

6. A method is described for the investigation of the reflex effect of stimuli arising in the upper air passages. A reflex to positive pressures is shown to occur which is similar to that produced by expansion of the lungs.

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THE SUBMAXIMAL RESPONSES OF THE SINGLE MUSCLE FIBRE.

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It has been shown by the writer [Gelfan, 1930, 1931], and also by Pratt [1930], that in the normal skeletal muscle stimulation of single fibres with micro-electrodes gives rise to submaximal contractions, graded according to the strength of the stimulus. The same results were obtained by Brown and Sichel [1930], and Asmussen [1932] on single fibres that had been dissected out. The following experiments were undertaken in order to determine whether these submaximal contractions differed in any way in their mechanical response from the maximal, all-or-none twitch, which results from stimulation with electrodes of larger area. It has already been shown by Gelfan and Bishop [1932] that the submaximal responses do not have action potentials, whereas in the identical fibre a characteristic diphasic action potential is obtained when it is stimulated to contract maximally. This is in harmony with the observations of Gelfan and Gerard [1930], and Hashida [1931] that the graded responses are not propagated throughout the length of the single fibre, and that the progressive spread of the localized response, as the strength of the stimulus is increased, is continuous only to a limited extent, after which the maximally conducted response is evoked. The maximal response is always accompanied by an action potential, whereas the graded submaximal contractions are not. These observations indicate not only that action potential and contraction are not inseparable, but that in any single striated fibre the former is of the explosive all-or-none type of mechanism whereas the latter is not. It has therefore been suggested by the author [1931], that when the muscle fibre responds maximally, whether it is stimulated indirectly or directly (electrically), it does so by virtue of the operation of the action potential mechanism.

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That the contractile mechanism might be directly stimulated without at the same time initiating the "excitatory change" was already recognized by Lucas [1907, p. 273]. He considered this possibility when he found that the local cathodic contraction, in contrast to the propagated response, was independent of the current gradient. Contractures also do not have wave-like action potentials [Gasser, 1930; Bremer, 1932]. The submaximal contractions as obtained by microstimulation might of course also be considered as of the nature of a contracture or local cathodic contraction. The latter, however, are characterized by the fact that they persist as long as the stimulating current is flowing. In the experiments here reported, therefore, records were obtained of the contraction curves of the submaximal and maximal responses of single fibres in order to compare their time relations, the stimuli being either induction shocks or constant currents of long duration.

Method.

The frog's (*Rana temporaria*) excised sartorius was used for all of the experiments here reported, prepared with a sufficient amount of tissue from the opposite leg, by means of which it could be suitably pinned on the glass slide provided with a piece of cork cemented to it. The tendon at the tibial end was tied with a thread which supported a small weight. The preparation under the microscope could be satisfactorily viewed by transmitted light, and any single fibre on the surface of the muscle could be clearly made out. The muscle was surrounded with cotton wool soaked with Ringer's solution, and was frequently irrigated with fresh solution.

The electrodes used were similar to those previously used by the author. Either platinum wire drawn out inside quartz capillaries to a few μ in diameter, or quartz capillaries of the same dimensions filled with frog's Ringer solution were used. The latter type, before filling with the Ringer's solution, are cemented into glass tubes of suitable dimensions. The glass tubing is then filled with Ringer's fluid and the latter is forced into the capillary to the very tip by means of pressure. An Ag-AgCl coil is then placed into the glass tube. The electrode is moved and oriented under the microscope by means of a Chambers' micromanipulator. Only one micro-electrode was used directly in contact with the fibre, the other indifferent electrode being an Ag-AgCl coil about 4 cm. long, placed near the muscle and covered by the same cotton wool that surrounded the muscle. The total diameter of the micro-electrode being only a few μ in diameter, it is possible to observe and record as near the point of stimulation as desirable.

The method of recording was essentially that of Pratt and Eisenberger [1919]. The preparation was sprayed with minute mercury droplets which were illuminated by a bright source of light from the side. The point of light that is reflected through the microscope by one of the mercury droplets on the responding fibre passes through a horizontal side eyepiece, and then to the motor-driven camera 1 ft. away from the microscope. The side eyepiece is so adjusted that the point of light from the mercury droplet is in focus on the camera film when the fibre is in focus for the observer. While the mercury globule is illuminated when recording, the responding fibre is also continuously observed with transmitted illumination.

Although the muscle was weighted by about 3 g. only, the contractions took place under fairly isometric conditions, since a single fibre had to contract against the resistance of the 400 fibres of the sartorius. No movement can be detected without magnification when only one fibre is responding.

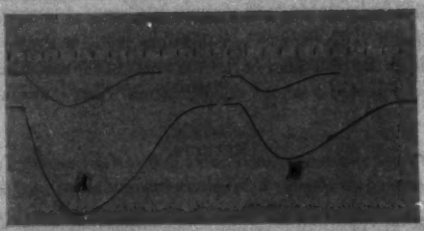
Results.

Responses to single induced shocks.

The essential condition for obtaining submaximal contractions in the single uninjured muscle fibre, as has already been pointed out [Gelfan, 1930; Gelfan and Gerard, 1930], is that the electrode should be of sufficiently small size. Asmussen [1932] obtained graded responses in the single fibre without the use of micro-electrodes. But in his experiments a single fibre was dissected out, and such an operation is scarcely possible without some injury to the fibre. Graded responses can be obtained in the injured fibre with diffuse stimulation [Hintner, 1930], and also in hearts that are in poor condition [Carlson, 1906]. In the curarized preparation, as already shown by the writer [1931], microstimulation is also necessary in order to obtain submaximal responses. This point is of some importance since Asmussen [1932, 1933], accepting the views of Lindhard [1932], considers the all-or-none behaviour of the fibre as a property of the end-plate. If this were true, microstimulation would not be necessary in the curarized preparation in order to obtain graded responses in the single fibre. Curarized preparations were at first used in the present experiments but later abandoned, as no advantage was found in the curarized over the non-curarized muscle. Not only do the micro-electrodes permit very fine localization of the stimulus, but they were also applied at the extreme pelvic (nerve-free) end of the muscle.

Plate I is a continuous record showing graded contraction curves of a single fibre. The stimulus was graded by movements of the secondary

coil. The current in the primary was delivered at intervals by a metronome interrupter, with condensers across the mercury cups to eliminate sparking. The duration of the submaximal responses, as may be observed from the record, is no longer than that of the maximal (last two responses in the record). Indeed the smaller responses are actually of shorter duration, approaching that of the maximal twitch as the magnitude of the mechanical response approaches that of the entire fibre. If the time relations of the contractile process at any point on the fibre are the same in a submaximal as in a maximal contraction, the longer



Text-fig. 1. A. Camera lucida drawings of a maximal and submaximal twitch of a single fibre stimulated by induced shocks, in which the shape and form of the contraction curves and time relations may be compared. Taken from Plate I, magnified ×4; total magnification ×700. B represents the same for the responses to constant current stimuli of 0·6 sec. duration taken from Text-fig. 3. Time signal above 0·01 sec. The record of the responses to the induced shocks was chosen in this case because of the unusual magnitude of the mechanical response of a single fibre. The other records of responses to induced shocks are more of the order of magnitude as represented by the responses to constant current.

duration of the maximal responses observed is not surprising since the activity is conducted over the entire length of the fibre, whereas in the submaximal response only a fraction of the fibre is involved. Apart from this difference in duration, the shape and form of the maximal and submaximal curves, as may be observed in Text-fig. 1, are essentially the same. The duration and magnitude of the response may vary somewhat in different experiments, depending upon the condition that the preparation is in, length of the muscle, mechanical conditions during any given experiment, etc. But whatever the duration, or shape and form of the maximal response may be under any given experimental condition, the relation between any given fraction of the maximal mechanical response and the maximal response is always of the kind shown in Text-fig. 1.

Responses to constant current.

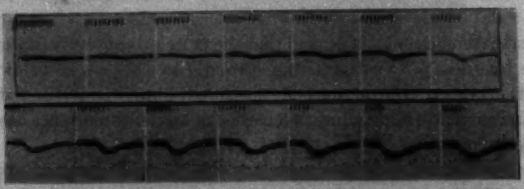
The responses of a skeletal muscle fibre to constant current, as Biedermann [1895, p. 160] demonstrated, may be of three types: a simple twitch, a twitch followed by a persistent shortening, and a persistent contraction not preceded by the twitch. The local cathodic contraction, as figured by Lucas [1908], which lasts as long as the exciting current does, is obviously the same as the third type mentioned by Biedermann. Reversible contracture type of responses to direct and indirect electrical stimulation have also been described by Bremer [1932]. Although the graded responses of the single fibre to induced shocks, as shown above, have time relations no longer than that of a twitch, it might still be argued that the submaximal responses are all examples of the local cathodic contraction and that the twitch-like brevity is permitted by the briefness of the stimulating current. As will be seen below, however, the graded contractions of the single fibre when stimulated by constant currents of long duration can be of any of three types described by Biedermann.

To avoid confusion, it is necessary first to point out the apparent reversal of polar effects with constant currents when micro-electrodes are used. The electrode system consisted of one micro and one large indifferent electrode. With this arrangement there is an apparent reversal of Pflüger's law, for the response occurs at the closure of the current when the micro-electrode is anode, and at the opening when it is cathode. The explanation of this phenomenon probably lies in the well-known fact that a localized electrode applied to a tissue induces an electric pole of opposite sign in the neighbouring parts of the tissue, and it would seem that in the conditions described this secondary pole is more effective than the primary.

Of the three types of response to constant current mentioned above, the twitch is of greatest interest in this case. The occurrence of submaximal twitches definitely demonstrates that in the single fibre graded contractions of twitch-like duration may be obtained with constant currents that far outlast the duration of the response. Plate II shows a series of responses graded by increasing the strength of a constant current by means of a potentiometer in the circuit. The micro-electrode in this case is the anode and the contractions are only at the make of the current. The duration of the responses is of the order of that of a twitch, although the stimulus is of about 0·5 sec. duration. Similarly, Text-fig. 2 shows responses at cathode to the break of the current. The last responses in

both cases are maximal, as further increase in stimulus strength did not increase the height of the mechanical response (sufficient increase in stimulus strength with consequent spread of current evokes additional units to respond maximally with the well-known step-like gradation). The shape and form of the submaximal contractions are not significantly different from the maximal ones, except for the magnitude of the response. As the camera lucida enlargements in Text-fig. 1 demonstrate more clearly, the duration of the submaximal response is actually shorter than the maximal twitch.

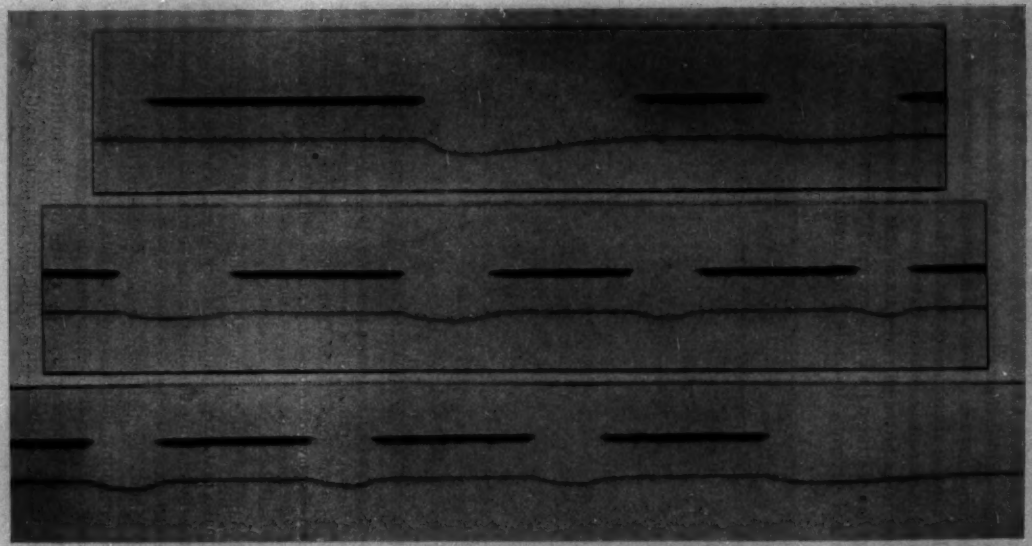
The response at the break of the current can be graded not only by the variation of the current intensity but also by grading the duration of the



Text-fig. 2. Graded responses of twitch-like duration to break of constant current at cathode. To be read from left to right and from above downward. Non-polarisable electrode 10 μ in diameter. Stimuli, lasting 0·6 sec., and graded by potentiometer, were delivered every 2 sec. The end of the time marker (0·01 sec.) above myogram in each strip of the record represents the break of the current. Most of the record between each make and break has been cut out, each strip representing only a small fraction of the entire duration of the stimulus. Operation of switch as in Text-fig. 2. Temp. 17° C. Microscopic magnification × 170.

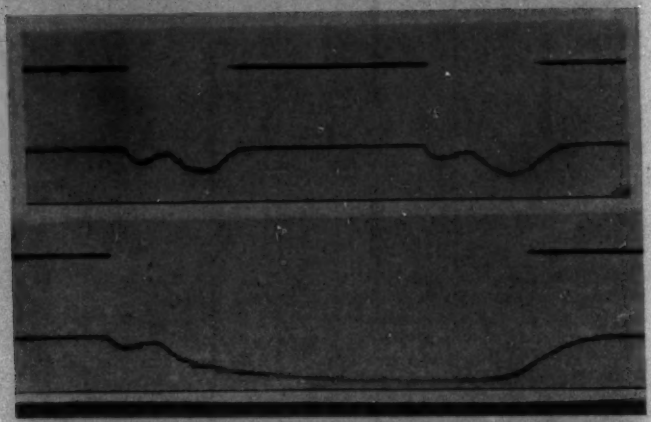
stimulus. With a current duration of about 1 sec., and an intensity that is maximal for this duration for the single fibre, fractions of the maximal response may be obtained by decreasing the duration and keeping the intensity constant. This is true for both the twitch-like responses and the sustained contractions. The latter effect may be seen in Text-fig. 3. A similar observation on the effect of duration of a tetanizing current of constant intensity giving sustained effects has already been observed by the author [1930].

The galvanic contractures in the single fibre were obtained both at the make of the current when the micro-electrode was the anode, in which case the response lasted as long as the current was on, and at the break of the current when the micro-electrode was the cathode. In the latter case, persistent contractions, some of which lasted several seconds, were



Text-fig. 3. Continuous record of graded contracture responses of a single fibre to break of constant current at cathode. Non-polarizable electrode 15μ in diameter. Read from left to right, from above downward. Time marker (0·01 sec.) indicates duration of stimulus, make at left or beginning of signal, and break at end of signal. Demonstration of inhibiting effect of make of current on contraction produced by the break at cathode. Temp. 16° C. Microscopic magnification $\times 170$.

obtained and these always began only after the cessation of the current. Text-fig. 3 shows graded contractures of the single fibre elicited by the break of the current at the cathode. The active inhibitory effect of the make of the current after the contracture has started as a result of the break of the current is well illustrated here, and also in Text-fig. 4. The inhibitory effect of the current on a muscle in a state of excitation was long ago demonstrated by Biedermann [1895] on the heart and on the veratrinized sartorius. Recently it has again been demonstrated by



Text-fig. 4. Persistent maximal contraction preceded by twitch at break of current, and inhibition by the make. Cathode 15 μ in diameter. Only one fibre responding, showing greater degree of shortening of maximal contracture as compared to maximal twitch. End of signal above myogram is break of current, beginning is the make. Temp. 16° C. Time below 0·01 sec.

Bremer [1932]. In the experiments of the last two investigators it is the anode make that exerts the inhibitory effect, whereas in our case, because of the reversal of polar effects with the micro-electrode, the effect is at the cathode make.

Text-fig. 4 shows the contracture response preceded by a twitch. Only one fibre is responding, but the twitch in this case is already maximal. The response is at the break of the current at the cathode. In the upper portion of the record the contracture after the twitch is inhibited before its full development by the making of the current again, shortly after the

response began. In the lower portion of the record, however, where the fuller development of the persistent contraction is permitted before the current is made again, the very much greater shortening may be observed. Visually, the maximal contracture response to the break of the current at the cathode is strikingly similar to a tetanus or to the type of response evoked by pricking a muscle fibre of the retrolingual membrane, as reported by Gelfan and Bishop [1933].

Discussion.

It is clear that submaximal contractions may be obtained in the single fibre that, in their time relations, are in no way different from a twitch. This is equally true for constant current stimuli as it is for induced shocks. Furthermore, it is possible to differentiate, in the submaximal responses of the single fibre as well as in the all-or-none responses of the fibres as a whole, contracture from twitches. The graded twitch-like responses in the single fibre, therefore, cannot be regarded simply as local cathodic contractions, as Ritchie [1932] suggests. The submaximal twitches, however, do differ from the maximal ones at least in one important respect. They are not accompanied by an action potential [Gelfan and Bishop, 1932][1]. Also, they are not propagated throughout the length of the fibre, and need a stimulus of restricted area.

The question as to the validity of the all-or-none principle has now become mostly a matter of definition. There is an all-or-nothing relation between stimulus and electric response in the muscle, and presumably between the stimulus and the propagated disturbance, but the contractile process is fundamentally capable of continuous gradation. This further indicates that contraction and electric response are distinct mechanisms. The fact that the two responses begin simultaneously [Roos, 1932] does not preclude their separability.

[1] It is difficult to accept the claim of Henriques and Lindhard [1920] and Lindhard [1932] that a muscle stimulated directly (by large electrodes, so that many or all fibres are contracting maximally) does not have an action potential. Action potentials have frequently been measured from muscles completely curarized, in which case there was no question of stimulating intramuscular nerve fibres. Adrian and Owen [1921] were able to demonstrate action potentials in the denervated gastrocnemius and sartorius. Henriques and Lindhard [1923] claimed that the action potentials in the last experiments were due to stimulus escape, but Adrian [1925] published further records from denervated muscle in which the form of the response was shown to be quite different from that of the stimulus escape. Records can easily be obtained with the Matthews's oscillograph showing both the stimulus escape and action potential, the former some distance from the latter, from a completely curarized sartorius. Furthermore, spontaneous discharges from a muscle in NaCl can be measured when the preparation is curarized [Adrian and Gelfan, 1933]. There is no question of stimulus escape in this case.

All the events that can occur when a muscle fibre is adequately stimulated are not interdependent to the degree that they must either all occur or not at all. Whatever the structural basis of the various processes may be, it is obvious that the fibre can be stimulated to give a slow response (reversible contractures) or a rapid one (twitch), and that the electric response may or may not accompany the mechanical one. Furthermore, the different processes involved do not necessarily obey the same laws. The all-or-nothing behaviour which characterizes the action potential mechanism is not a fundamental property of the contractile process, as is indicated by the various types of contracture and by the submaximal responses of the single fibre.

Summary.

Records have been made of the contractions of single muscle fibres of the frog's sartorius which permit analysis of the duration and shape and form of the mechanical response to induction shocks and constant current of long duration. The graded submaximal responses in the single fibre could thus be compared to the maximal ones in the same fibre.

All of the three types of responses that can be produced in the muscle as a whole by diffuse stimulation with constant current can also be duplicated in the submaximal responses of the single fibre. The rapid submaximal responses to constant current in the single fibre have the time relations of a twitch.

There is an apparent reversal of polar effects when a micro-electrode and a large indifferent electrode are used for stimulating.

A contracture produced by the break at the cathode can be actively inhibited by the make of the current again.

I wish to acknowledge my indebtedness to Profs. E. D. Adrian and J. Barcroft for all the courtesies extended to me during my stay at this laboratory as a Guggenheim Memorial Fellow.

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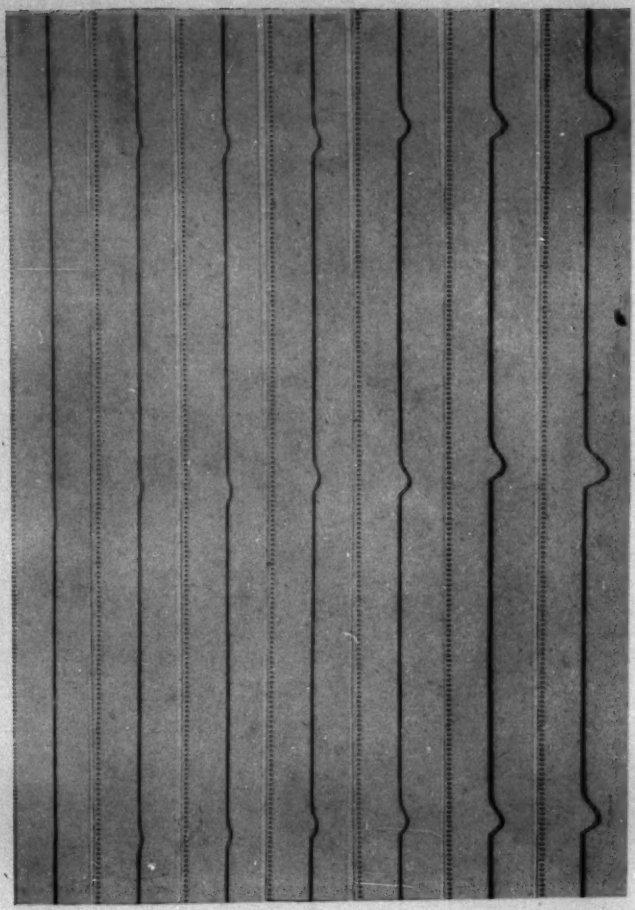

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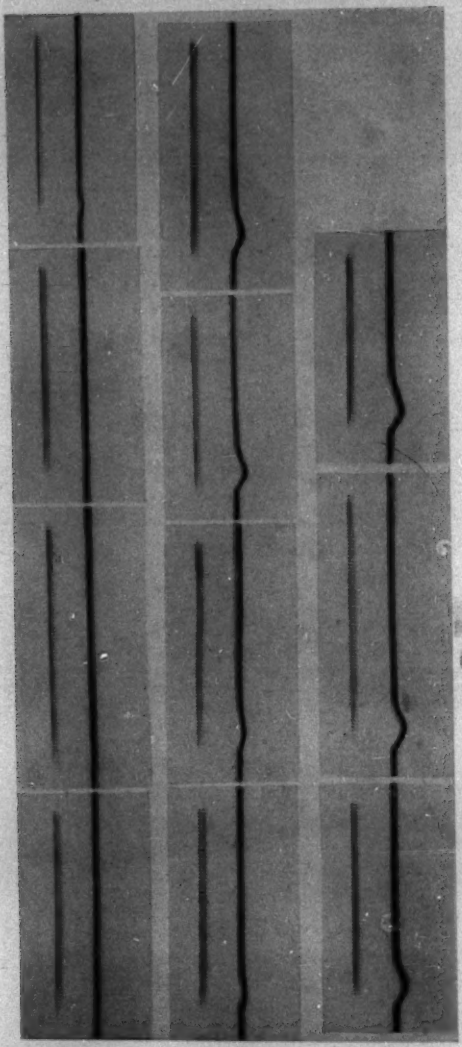

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EXPLANATION OF PLATES I AND II.

Plate I.

Continuous record showing graded responses of a single fibre in the sartorius muscle to single induction shocks, increasing progressively in strength. Record reads from left to right and from top to bottom. Last two contractions are maximal. Time signal at top of record 0·01 sec. Unipolar stimulation with non-polarizable electrode 5 μ in diameter (see also text). Microscopic magnification × 170. Temp. 16° C.

Plate II.

Graded responses of twitch-like duration to constant current stimuli. To be read from left to right and from top to bottom. The non-polarizable electrode, 15 μ in diameter, was the anode, and responses are only at the make. Stimuli lasting 0·5 or more seconds were graded by potentiometer and delivered every 2 sec. Temp. 16° C. Time 0·01 sec. Microscopic magnification × 170. The beginning of the time signal at the left in each case indicates the make of the current, and the end on the right the break of the current. A hand key of a double-pole mercury cup switch made and broke contact of both the stimulating current and the current illuminating mirror on vibrating tuning fork. The imperfect relation between beginning of stimulus and beginning of time marker is due to the fact that the contacts in the two mercury cups were not made perfectly simultaneously.

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THE BEHAVIOUR OF THE LIVER GLYCOGEN DURING DECEREBRATION HYPERGLYCÆMIA AND THE INFLUENCE OF ATROPINE AND OF ERGOTAMINE ON THIS CONDITION.

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(*Received September* 27, 1933.)

In the experiments of Donhoffer and Macleod [1932] on decerebration hyperglycæmia, it was shown that atropine injected in repeated doses of 1 mg. was capable of preventing or at least retarding the rise in the blood sugar percentage in fasted rabbits in which the vagus nerves were cut. In well-fed animals with large stores of liver glycogen, on the other hand, atropine and vagotomy had no influence on the development of the hyperglycæmia. In these experiments the influence of ergotamine was undecisive because of difficulties in the collection of blood from the ear vein. Since it is important in solving the problem of the nature of the nerve control of the blood sugar level to know exactly the effect of these two drugs on decerebration hyperglycæmia, it was considered advisable to reinvestigate their effects with the difference that the vagus nerves are left intact. The results of such experiments are shown in Tables I and II. In another series of experiments, Donhoffer and Macleod found that the degree of hyperglycæmia following decerebration was not related to the percentage amount of glycogen present in the liver to start with. They also found that the amount of sugar arising from the glycogen which disappeared from the liver during the course of a decerebration experiment on a previously fasted animal was far less than that required to cause a comparable increase in the blood sugar percentage when given by continuous intravenous injection to rabbits under amytal. This led to the conclusion that the nerve disturbance set up by the decerebration must excite a new formation of sugar in the liver—a secretion, as Claude

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Bernard thought—and that the glycogen found present in the organ was merely a by-product of this process which might either become increased or decreased in amount during an experiment. Even supposing that over-production of sugar in the liver, rather than decreased utilization in the tissues, is the cause of the hyperglycæmia which follows decerebration, it is obvious that only a very approximate estimate of the amount of extra sugar, which would be required to produce it, could be obtained by seeing how much glucose had to be administered by continuous intravenous injection into a normal animal to bring about a corresponding rise in the blood sugar level. Quite apart from a possible effect of anæsthesia, differences in the two animals—the decerebrated and the anæsthetized—might depend on the state of carbohydrate metabolism at the time of the experiment; whether the muscles, for example, were building up glycogen or breaking it down. Such comparisons would only be justifiable when changes in the glucose, glycogen and lactic acid content of the entire body were taken into account, and even then one would have to be certain that the same amounts of carbohydrate were being metabolized by the two animals under comparison.

Although there is, therefore, no way of knowing how much extra sugar is necessary to cause a degree of hyperglycæmia comparable with that following decerebration, it was considered important to ascertain how the percentage of liver glycogen would behave from hour to hour when large amounts were present at the time of decerebration. If any degree of parallelism could be detected between the rate at which the percentage of sugar rises in the blood and that at which the glycogen percentage falls in the liver, it would be strong presumptive evidence that over-production of glucose out of preformed glycogen in this organ is mainly responsible for the hyperglycæmia which follows decerebration in well-fed animals. It is quite certain that extra sugar production out of preformed glycogen in the liver is at best only a secondary factor in causing decerebration hyperglycæmia in fasted animals, but this does not rule out the possibility that this is the main source of the sugar in well-fed ones with much stored glycogen in the liver. This increased glycogenolysis might conceivably last until all the preformed glycogen has been used up and then be followed by increased gluconeogenesis.

The methods used for decerebration, the removal of material for analysis and the determination of glycogen, blood sugar, etc. were the same as those described by Donhoffer and Macleod. The only experimental detail that requires mention here concerns the choice of animal and its feeding prior to fasting. From various laboratories, as well as this

TABLE I. Effect of different amounts of atropine on the percentages of blood sugar and liver glycogen after pontine decerebration.

Exp. No.	Wt. of rabbit (kg.)	Intravenous injections of atropine (mg. per kg.)†	Blood sugar (mg. per 100 c.c. blood) Initial value	½	1	1½	2	2½	3	3½	4	4½	5	5½	6	6½	Liver glycogen Initial	Final
16	1·9*	(1) 1·05 (2) 1·05 (3) 1·05	130	160	180	180	190	225	240	255	295 (1)	325	390 (2)	440 (3)	—	490	6·9	2·8
18	1·3	(1) 2·4	225	—	190	145	200	250	345	435 (1)	410	390	375	365	340	—	8·9	1·8
20	0·8	(1) 5·3 (2) 10·6	155	—	225	270	280 (1)	—	335	395	480	410 (2)	365	375	370	390	12·6	6·2
22	1·4	(1) 4·5 (2) 4·5	145	—	210	210	165	170	—	280 (1)	245	250	220	210 (2)	—	200	0·5	0·1
24	0·9	(1) 10	180 (1)	155	145	135	165	175	175	390	265	265	290	290	450	—	10·9	6·6
26*	0·8	(1) 11	145	—	170	220	225	230	230 (1)	340	300	325	330	350	380	—	8·1	2·3
27*	1·3	(1) 6	250 (1)	250	160	230	205	205	215	290	260	305	340	—	—	—	0·6	0·1

* Animals fed high carbohydrate diet and not fasted, except Nos. 26 and 27, which were fasted overnight.

† The injections were made at the time intervals indicated by the numbers. The amount of atropine given at each injection is also given in this column.

one, the results of recent years have shown that the behaviour of carbohydrate metabolism under any particular condition, such as administration of insulin or adrenaline, fasting and even such violent disturbances as those caused by decerebration, may be influenced by the age of the animal, the season of year, the time of day, as well as by the previous feeding. In order to obtain consistent results even in decerebration experiments, it is therefore necessary that the experimental conditions be rigidly standardized. This has been done as far as possible in the present investigation, and would have been done completely had a suitable supply of rabbits been available.

(1) *The effect of atropine.* (Table I.)

In one of the animals (No. 27) the liver glycogen to start with was low, as a result of previous fasting, and the atropine given at the time of decerebration retarded although it did not prevent the development of hyperglycæmia. There is no doubt, however, from the results of Donhoffer and Macleod, that atropine under these conditions does usually prevent pontine decerebration from having any effect on the blood sugar level. On the other hand, when the atropine is given to carbohydrate-fed animals having large amounts of liver glycogen, it does not retard the rise in the blood sugar level following decerebration unless the dose be excessive (10 mg.), and even then the retardation is only temporary. In Exps. 20 and 22, in which atropine was given after the hyperglycæmia had become established, only a doubtful depression followed in the blood sugar level, and in Exp. 26, in which as much as 11 mg. was injected, the hyperglycæmia was not affected. On the other hand, when a large dose of atropine (10 mg.) was given at the same time as decerebration was performed, as in Exp. 24, it did distinctly retard the development of the hyperglycæmia. Doses of atropine less than 5 mg. had no effect, as is seen in Exps. 16 and 18.

As might be expected, the atropine did not influence the break-down of liver glycogen.

(2) *The effect of ergotamine.* (Table II.)

In the experiments of Donhoffer and Macleod the results obtained after injecting ergotamine were not conclusive, because of the difficulty of collecting blood from the ear veins. It was decided to repeat these experiments by collecting the blood from the carotid artery. Stock-fed animals

TABLE II. Behaviour of the percentages of sugar and lactic acid in the blood, and of glycogen in the liver after intravenous administration of ergotamine alone and of ergotamine plus atropine and vagotomy following decerebration.

Exp. No.*	Wt. of rabbit (kg.)	Region of decerebration	Injection amount (mg. per kg.)‡	Constituent determined	Blood (mg. per 100 c.c.) Initial value	Time after decerebration (hours) ½	1	1½	2	2½	3	3½	4	Liver glycogen p.c. Initial	Final
57	1·3	II	(*b*) 0·4	Sugar	185	120	140	115	115	128	135	140	165	4·0	1·8
				L. acid	67				121				116		
58	1·0	III	(*b*) 0·5	Sugar	138	174	174	165	178	153	151	163	203	4·3	3·3
				L. acid	45			64					62		
61	1·7	III	(*b*) 0·5	Sugar	143	115	125	138	130	125	150	140	125	2·7	3·2
				L. acid	20				30				40		
63	1·9	III	(*b*) 0·5	Sugar	163	158	163	205	228	205	190	193	155	2·3	0·8
				L. acid	36				202				87		
64	1·3	III	(*b*) 0·8	Sugar	135	110	105	130	120	125	155	160	225	0·8	0·8
				L. acid	30				30				39		
65	1·1	III	(*b*) 0·9	Sugar	125	150	120	150	135	130	170	140	180	3·6	3·9
				L. acid	84				33				37		
51	1·1†	III	(*a*) 10	Sugar	165	180	130	120	105	105	125	140	170	5·5	3·0
			(*b*) 0·5	L. acid	32				93				93		
54	1·6†	III	(*a*) 10	Sugar	203	235	197	151	183	151	193	213	193	2·2	3·8
			(*b*) 0·6	L. acid	—				64				95		
56	1·8	III	(*a*) 10	Sugar	160	159	120	150	163	163	133	108	103	0·3	0·5
			(*b*) 0·6	L. acid	63				108				106		

* Stock fed and fasted overnight, except in No. 65, which was maize fed and not fasted.
† Vagus nerves cut.
‡ (*a*) indicates atropine. (*b*) indicates ergotamine.

fasted for variable periods were mainly used, so that the percentages of glycogen in the liver varied from animal to animal. There are three experiments (Nos. 58, 61 and 63) in which the animals received 0·5 mg. ergotamine at the time of decerebration in region III, and in all of them the blood sugar level rose not at all or much less steeply than usual. In two experiments (Nos. 64 and 65) larger doses of ergotamine (0·8–0·9 mg.) still more markedly suppressed the hyperglycæmia. The behaviour of the blood lactic acid is of some interest in these experiments, for in three out of the five its percentage after four hours had scarcely risen above the initial value. With regard to the behaviour of the liver glycogen, it will be observed in four of the experiments that the percentage had scarcely changed after four hours. This is a very different result from that obtained following decerebration when no drugs are given and is probably of some significance. The table also contains a control experiment in which decerebration was performed in region II; the blood sugar percentage remained constant, the blood lactic acid percentage rose sharply and the liver glycogen percentage fell.

The results taken as a whole leave no doubt that ergotamine alone can completely counteract the effects of pontine decerebration in fasted stock-fed rabbits. This result is of interest in view of that obtained by Nitzescu and Munteanu [1932], who found that ergotamine prevents the development of hyperglycæmia following adrenaline.

The table also shows the results of three experiments (Nos. 51, 54 and 56) in which ergotamine (0·5–0·6 mg.) was injected along with atropine (10 mg.) into rabbits that had fasted overnight after being stock fed. In two of these animals the vagus nerves were also cut. The results with regard to the blood sugar level and the liver glycogen percentages are the same as after ergotamine alone, but they differ with regard to the blood lactic acid, which rose sharply. Apparently atropine annuls the effect which ergotamine has in preventing increase in the blood lactic acid percentage, but we do not wish to put much emphasis on this particular observation, since it is quite evident that, in rabbits at least, the blood lactic acid may vary considerably from unknown causes.

(3) *Behaviour of glycogen at intervals following pontine decerebration.* (Table III.)

In four experiments on fed animals (Nos. 31, 33, 34 and 41) decerebrated in region III, the blood sugar level rose in the usual manner, so that decided hyperglycæmia was present by the second hour. Meanwhile, the

TABLE III. The behaviour from hour to hour following decerebration of the percentages of sugar in the blood and glycogen in the liver and muscles.

Exp. No.	Wt. of animal (kg.)	Condition of animal	Level of decerebration	Blood sugar percentage: Before decerebration	Hours after decerebration: ½	1	1½	2	2½	3	3½	4	Glycogen percentage: Time of taking sample	Liver	Muscle
31	1·3	High carbohydrate	III	215	180	190	235	275	375	395	485	430	Initial	4·9	0·5
													1st hr.	5·2	—
													2nd hr.	4·1	—
													4th hr.	2·1	0·4
32	1·8	Same	II	110	135	120	100	110	140	210	285	295	Initial	8·9	0·8
													2nd hr.	7·3	—
													3rd hr.	5·6	—
													4th hr.	6·2	0·7
33	1·7	Same	III	135	170	170	180	200	285	355	345	350	Initial	2·2	0·4
													2nd hr.	2·6	—
													3rd hr.	1·3	—
													4th hr.	0·3	0·2
34	2·2	Same	III	180	215	275	350	365	465	490	620	560	Initial	12·7	0·7
													2nd hr.	11·7	—
													3rd hr.	5·9	—
													4th hr.	5·5	0·4
41	1·5	Same (renal vessels ligated)	III	175	250	255	345	375	390	400	425	350	Initial	4·4	0·4
													1st hr.	2·2	—
													2nd hr.	1·8	—
													3rd hr.	1·0	—
													4th hr.	0·3	0·1
35	2·3	Same (renal vessels ligated), atropine 10 mg. injected at (*a*)	III	200 (*a*)	340	325	435	460	490	510	—	470	Initial	11·9	1·0
													1st hr.	6·0	—
													2nd hr.	2·8	—
													4th hr.	2·7	0·8
37	1·5	Fasted atropine at (*a*)	III	160 (*a*)	280	255	195	170	220	245	320	350	Initial	0·5	0·8
													1st hr.	0·3	—
													2nd hr.	0·3	—
													3rd hr.	0·2	—
													4th hr.	0·0	0·5

liver glycogen percentage remained practically unchanged in three out of the four experiments. After the second hour the rise in blood sugar percentage became very marked and the liver glycogen percentage rapidly decreased. There is evidence to show that the rapid increase in blood sugar in the later stages of the experiments was partly due to the handling of the liver entailed in removing pieces for analysis; thus, a similar rise occurred in an animal decerebrated in region II (No. 32). Administration of atropine at the time of decerebration (No. 35) did not prevent the development of hyperglycæmia when the glycogen percentage was high, but delayed it when this was low (No. 37).

The behaviour of the glycogen in the liver in these experiments confirms the conclusion previously drawn by Macleod and Donhoffer that the course and degree of decerebration hyperglycæmia is little dependent upon the rate at which it is discharged as glucose into the hepatic veins. No doubt, other things being equal, the percentage of blood sugar will tend to rise somewhat higher and to remain high for longer in glycogen-rich than in glycogen-poor animals, but this difference is by no means sufficiently pronounced to indicate that increased hepatic glycogenolysis is the function primarily responsible for the hyperglycæmia. For example, in Exp. 32 of the present series, in which decerebration was performed in region II, the blood sugar level did not change until after 2½ hours—when handling of the liver began to affect it—yet the fall in the liver glycogen percentage, up to 2 hours at least, was as great as in any of the other experiments.

As was to be expected, the percentage of glycogen in the leg muscles fell in all the experiments, and although this may have occurred in some more rapidly than in others, no relationship can be detected between the rate of this decline and that of the liver glycogen or between it and the rate at which the blood sugar percentage increased.

Conclusions.

1. When the percentage of liver glycogen is high in rabbits, atropine has only a slight retarding effect on the development of decerebration hyperglycæmia.

2. Under the same conditions, and also when the glycogen is low, ergotamine almost entirely prevents the hyperglycæmia.

3. In the majority of the experiments with ergotamine no increase occurred in the percentage of blood lactic acid following decerebration and the liver glycogen remained unchanged.

4. No parallelism could be demonstrated between the rate at which glycogen disappears from the liver following decerebration and the increase in blood sugar percentage.

The author wishes to thank Prof. J. J. R. Macleod for his advice and assistance during this investigation, which was undertaken at his suggestion.

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THE EFFECT OF DIET, OF INSULIN AND OF THYROXINE UPON THE ADRENALINE CONTENT OF THE SUPRARENAL GLANDS.

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(*Received October 11th*, 1933.)

The effect of diet.

In a recent communication Rosenblueth and Gayet [1932] report that rats placed on meat, fat or carbohydrate diet show no difference in their adrenaline content, and they conclude that the adrenaline store of the suprarenal glands is unaffected by the character of the diet. Being unaware of the work of these authors, I have been conducting similar experiments with, however, a somewhat different result.

In order to obviate any possible effect which climatic conditions might have on the results of the experiments, the investigation was carried out on three groups of animals, one during the hot Egyptian summer (all males), the second during the autumn (females and males), and the third during the beginning of winter (all females). The results obtained with these three groups showed no difference, and they will therefore be described together. The experiments were performed on 105 white rats of approximately the same weight and age; they were not fully grown, as it was hoped that the effect of diet would be more conspicuous in the growing animal. Before placing the animals on the different diets, in order to equalize their condition, they were kept for 7 days on a mixed diet composed of 68 p.c. dried bread, 20 p.c. meat containing fat, 10 p.c. milk and 2 p.c. salt mixture, all mixed into a paste with the addition of water.

On the 8th day the rats were separated into five batches of twenty-one rats each. The first batch continued to receive a mixed diet; the second was fed on lean minced meat which was slightly cooked for about 40 min. in thin layers in a hot-air oven; the third batch received mutton fat, which was separated from cellular elements by being melted on a water bath; the fourth batch was fed on raw rice, and the fifth was starved. All

the rats received a plentiful supply of water, and all except the fifth batch were given 2 g. of salt mixture and 5 c.c. of cod-liver oil to every 100 g. of the diet.

One third of the animals in each group was killed on the 9th–10th day of receiving the special diet, another third was killed on the 15th–16th day, and the remainder on the 20th–22nd day. Of the animals which were starved, some were killed on the 9th day, and all the rest on the 15th day. The animals were killed in an atmosphere of chloroform and coal gas [Crowden, 1929]. They died within 1–2 min. The suprarenal glands were at once excised, freed from connective tissue and weighed, and the adrenaline content of both glands together was estimated. The usual method of the chemical estimation of adrenaline by hydrochloric acid extraction, recommended by Folin, Cannon and Denis [1912–13], has been criticized by Baker and Marrian [1927], who found that extraction with trichloroacetic acid gave more reliable results. Wiltshire [1931] found that the addition of a trace of alanine retards the oxidation of adrenaline in solution, a result which I can entirely confirm. In view of this, the adrenaline was estimated as follows:

Both suprarenal glands were rapidly and finely ground with silver sand, with the addition of a few drops of 10 p.c. trichloroacetic acid containing 0·25 p.c. of alanine. About 1·5 c.c. of the trichloroacetic-alanine solution were added after the grinding and the whole mixture was agitated, the sand and precipitated proteins were allowed to settle, and the supernatant liquid was filtered off through a little wad of glass wool. The residue was extracted once more with 1·5 c.c. of the solution, filtered off, and then washed twice with a few c.c. of distilled water and a few drops of the trichloroacetic-alanine solution. The filtrates were centrifuged, and the small residue was once more thoroughly washed and centrifuged. Finally the whole extract was diluted to 25 c.c. in a measuring flask. The estimation of the adrenaline in the extract was carried out colorimetrically in a Duboscq colorimeter with the addition of Folin's "uric acid reagent," the standard being a 0·04 mg./100 g. uric acid solution.

The results of the experiments are represented graphically in Fig. 1 which shows that, in spite of the fact that there are considerable variations in the adrenaline content of the individual rats in each batch, there is not much difference between the control rats and the rats kept on protein diet, while the adrenaline content of the rats kept on the carbohydrate diet and on the fat diet is considerably diminished. The starved rats also showed diminution of the adrenaline content, confirming the results of all

previous observers [Vincent and Hollenberg, 1920; Mouriquand and Leulier, 1928; Rosenblueth and Gayet, 1932].

The comparison of the weight of the suprarenal glands of rats kept on different diets and the relative amounts of adrenaline per g. of gland

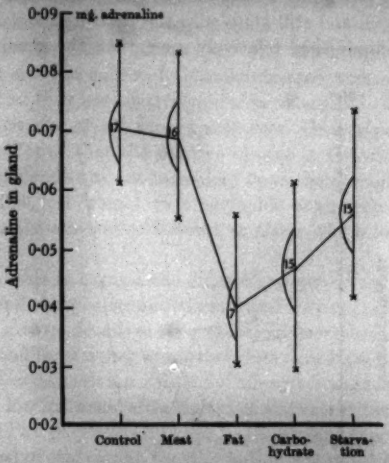

Fig. 1. The influence of different diets on the adrenaline content of the suprarenal glands. Vertical lines show the extent of the individual variations in the corresponding batches of rats. The averages are joined together. The figures in the segments represent the number of rats in each batch whose adrenaline content varies between the limits enclosed by the segment. There were twenty-one rats in each batch.

TABLE I.

	Weight of rats before diet g.	Weight of rats after diet g.	Weight of glands g.	Adrenaline content mg.	mg. adrenaline per g. gland	g. gland per kg. body weight
Control	2982	3276	0·745	1·459	1·958	0·227
Meat	2900	2976	0·708	1·373	1·939	0·237
Fat	2910	2289	0·519	0·837	1·595	0·227
Carbohydrate	3079	2512	0·393	0·991	2·525	0·156
Starvation	2940	1785	0·527	1·180	2·239	0·295

show additional points of interest. Table I gives the figures for the sum of all the rats in each group. On comparing the maxima and minima from the different groups, the result was found to be the same. The most striking difference is observed in the relative amount of adrenaline per g.

of gland in the case of fat, carbohydrate and inanition. In all three batches the weight of the suprarenal glands (col. 3) was found to be diminished as compared with the controls and the protein fed animals. With the fat diet, however, the diminution of the weight of the suprarenal glands was less pronounced than that of their adrenaline content, while during starvation and still more with the carbohydrate diet the weight of the glands diminished relatively more than the absolute amount of

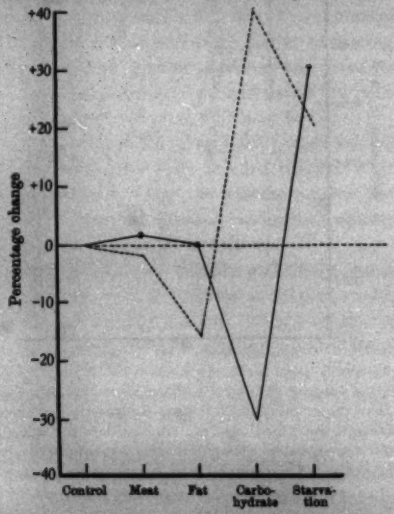

Fig. 2. The influence of different diets on the weight of the suprarenal glands in g. per kg. body weight (continuous line) and of their adrenaline store in mg. per g. of gland (dotted line). The calculation was made in comparison with the control animals.

adrenaline. Thus, with the fat diet, the adrenaline content per g. of gland diminished as compared with controls, while during starvation and even more with the carbohydrate diet it increased. These figures are of further interest when compared with the relative change of weight of the suprarenal glands per kg. body weight. In this respect there is no difference between the control batch and the protein fed rats, but in starvation the glands are relatively larger, and in the carbohydrate fed animals relatively smaller than in the controls. In the case of fat diet the weight of

the glands per kg. body weight does not differ from that of the normal. It is thus obvious that in the case of the fat diet the weight of the gland and that of the body diminished at the same rate, while in the case of the carbohydrate diet the glands diminished far more rapidly than the weight of the body. In starvation the weight of the body diminished more rapidly than the weight of the suprarenals.

Thus with the carbohydrate diet the suprarenals are markedly diminished in size but relatively heavily charged with adrenaline, while with the fat diet they are relatively poor in adrenaline. Fig. 2 represents the average results of all the experiments. On comparing individual animals it was found that the relative change in the adrenaline store of the suprarenal glands was not dependent upon the change in body weight, since rats which lost weight to a negligible extent showed as great a diminution in the adrenaline content as those which lost considerably more weight.

It is impossible to explain the difference between the results of these experiments and those of Rosenblueth and Gayet [1932], especially as these authors give no details of their experiments. It would be of interest to know the conditions under which their rats were kept, since the changes in weight of their animals are strikingly different from those of the present experiments. For instance, after 8 days of starvation the weight of their rats diminished by only 5·5 p.c., while in the present experiments, during the same period of starvation, the diminution was never less than 30 p.c. In the case of fat diet all the rats in the present experiments showed a diminution in weight, while the rats of Rosenblueth and Gayet either did not change in weight or, in the case of 35 days of feeding on fat, increased in weight.

The effect of insulin.

There are various data in the literature concerning the activity of the suprarenal bodies under the influence of insulin, but no determinations were made of the adrenaline content. Most of the observers [Sundberg, 1923; Houssay and Lewis, 1923; Lewis and Magenta, 1925; Artundo, 1925; Ricci and Hilami, 1925; Houssay, 1925; Tada, 1931] consider that extirpation of both suprarenal glands leads to an increased sensitivity of the animal to insulin. Some authors [Cannon, McIver and Bliss, 1924; La Barre and Houssa, 1932] report that insulin hypoglycæmia leads to an increased secretion of adrenaline, the effect of which is to restore the blood sugar level by the mobilization of glycogen. Stewart and Rogoff [1923 *a*, *b*] hold the view that the adrenaline production is

unaffected by insulin. Thatcher [1926] investigated the effect of prolonged daily administration of insulin on the weight of the suprarenal bodies, and found it almost unchanged; the adrenaline content, however, was not determined.

In order to determine the effect of insulin on the load of adrenaline in the glands, two sets of adult rats, ten in each, were treated as follows: into one set over a period of 10–15 days small, definitely subconvulsive, doses (2 units at each injection) of insulin were injected, into the other, convulsive doses (10 units). Burroughs Wellcome's or Schering's insulin was used. In every case a control batch of the same number of animals of exactly the same weight was injected with an equal volume of a physiological saline solution. The results of these experiments are summarized in Table II.

TABLE II.

	Weight of rats after experiment g.	Weight of glands g.	Adrenaline content mg.	mg. adrenaline per g. gland	g. gland per kg. body weight
Subconvulsive doses of insulin	1809	0·442	0·768	1·741	0·244
Control	1788	0·319	0·654	2·048	0·177
Convulsive doses of insulin	2050	0·423	0·323	0·772	0·206
Control	2050	0·434	0·868	2·000	0·212
Thyroxine	1856	0·512	0·848	1·656	0·276
Control	1883	0·327	0·661	2·021	0·174

In every case the rats treated with subconvulsive doses of insulin showed a larger adrenaline content than their controls. Since the weight of the suprarenal glands was increased to a greater extent than the amount of adrenaline, the adrenaline content per g. of gland was diminished. The difference in the adrenaline content in the suprarenals was not large, but was definite enough to exclude the possibility of being due to individual variations, especially as this increase in adrenaline, as well as the increase in weight, was found in every single experiment. It is tempting to suggest, in view of the researches of the above-mentioned investigators, that the increase in weight of the suprarenal bodies and of their adrenaline content following the administration of insulin is due to a state of hyperactivity.

In the second set of animals, in which convulsive doses of insulin were used, the suprarenal bodies were found to have lost a considerable amount of their adrenaline store, as shown in Table II. The weight of the suprarenal bodies remained the same. It is impossible to say whether the depletion of the suprarenal bodies was the result of hypoglycæmia or of the prolonged convulsions of the animals. In order to determine the

mechanism of the action of the convulsive doses of insulin on the suprarenal glands, the effect of insulin was compared before and after section of the splanchnic nerves. Elliott [1912] demonstrated in cats that the right and left suprarenal bodies contained almost the same amount of adrenaline. The present work shows that this also holds for white rats. Denervation of the suprarenals on one side was carried out, under A.C.E. anæsthesia, through an abdominal incision, the wound being carefully closed after operation. The animals recovered a few minutes after the operation, and in about 30 min. they showed no difference from normal rats. Insulin was injected 2–5 hours after the operation. The animals were killed 2–4 hours after injection, and the adrenaline content of each suprarenal was determined separately. The average amount of adrenaline found in the ten normal glands was only 54·5 per cent. of that found in the denervated glands. In control animals, into which after the same operation physiological saline solution was injected, the innervated suprarenal contained on the average 71·5 p.c. of the amount found in the denervated gland. This result confirms that of Elliott [1912] and shows that the operative procedures had a marked effect on the innervated glands. The innervated suprarenals of the animals which received insulin were, however, depleted to a much greater extent than those of the controls which received only saline.

In order to make the effect of the insulin on the suprarenals independent of the effect of the operation, the experiments were repeated with the following modification. In each animal the left suprarenal was denervated while the right one was removed. Some of the rats operated upon in this manner received convulsive doses of insulin while others were kept as controls and received physiological saline instead. Two to four hours after the injection the animals were killed. In the case of the control animals the adrenaline content of the two glands was, in all cases, almost equal. On the other hand the adrenaline content of the denervated glands of the rats which received insulin was considerably diminished, the denervated gland containing on the average 70 p.c. of that found in the gland which was extirpated before the injection. This diminution must be attributed to an effect which insulin or the hypoglycæmia exerts directly on the suprarenal bodies. Besides this direct peripheral influence, however, there is also an influence through the nervous system.

On comparing the effect of insulin upon the normally innervated suprarenals as given in Table II with the effect upon the denervated glands, we find that in the first case the glands discharged on an average

63 p.c. of their adrenaline content, while the denervated glands discharged only about 30 p.c. These results, which were invariably obtained in a large number of experiments, show that insulin exerts a double influence. It stimulates the suprarenals through the central nervous system along the splanchnic nerves as well as peripherally by acting upon the gland itself. This conclusion is supported by experiments in which both glands were left in the animals, but one was denervated while the other was left intact. In control animals the innervated gland contained about 70 p.c. of adrenaline as compared with the denervated side, while in the animals which received insulin it contained 50 p.c., showing that under the action of insulin it lost more adrenaline than from the operation alone. In these experiments both the innervated as well as the denervated suprarenals were left *in situ*, so that the difference in their adrenaline content was due to the central impulses reaching one gland and not the other.

The effect of thyroxine.

In view of the fact that the effect of thyroxine is in many respects similar to that of adrenaline, it was of interest to find whether repeated administration of thyroxine would lead to any change in the adrenaline content of the suprarenal glands. For this purpose ten normal rats were each given injections of 0·5 mg. of thyroxine for 10–15 days. Similarly ten other rats of exactly the same weight had injections of physiological saline solution. The animals were killed and the suprarenals were treated in the same way as before. The results of these experiments are given in Table II. In every case the animals that had thyroxine had heavier suprarenal glands which contained more adrenaline. But the increase in weight of the glands was proportionately greater than that of their adrenaline so that, when calculated per g. of gland, the adrenaline content was relatively diminished. Thus the effect of thyroxine on the suprarenals and their adrenaline store is similar to the effect of small subconvulsive doses of insulin.

Conclusions.

1. The adrenaline content and the weight of the suprarenal glands are affected by variations in the diet. While high protein diet has no marked effect, fat diet leads to a diminution of the absolute content of adrenaline and of the total weight of the glands. The relative amount of adrenaline per g. of gland is also slightly diminished. Carbohydrate diet also leads to a diminution of the absolute content of adrenaline; the weight of the suprarenals is strikingly diminished so that the relative amount of adrenaline per g. of gland is considerably increased.

2. Repeated administration of insulin in subconvulsive doses leads to an increase in the weight of the suprarenals and of their adrenaline content. In convulsive doses, insulin leads to a depletion of the adrenaline store. This effect of insulin is produced through the nervous system as well as by peripheral action on the suprarenal bodies.

3. Repeated injection of thyroxine also leads to an increase in weight and of adrenaline content of the suprarenal bodies.

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THE CORRELATION BETWEEN THE ACTION OF INSULIN AND ADRENALINE UPON THE MUSCLE AND LIVER GLYCOGEN.

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It was shown by Dudley and Marrian [1923] that the hypoglycæmia which follows an injection of insulin into a normal animal is accompanied by a mobilization of glycogen from the liver as well as from the muscles. The hyperglycæmia caused by injection of adrenaline is also associated with a depletion of the glycogen stores. There is, however, no agreement as to whether in the latter case it is the liver or the muscle glycogen which is chiefly affected. Ohara [1925] found that adrenaline brings about a hyperglycæmia in rabbits even after their glycogen store has been greatly reduced by starvation. Hyperglycæmia has also been produced in hepatectomized dogs, and in this case it was found that the glycogen store of the muscles was considerably diminished. Ohara also found that in normal dogs a single injection of adrenaline reduced the glycogen content of both the liver and the muscles. On the other hand, Cori and Cori [1928] state that, in fasting rats, adrenaline leads to an increase in the liver glycogen and a decrease in the muscle glycogen, an observation which was confirmed by Blatherwick and Sahyun [1929]. Goldblatt [1929] finds that adrenaline in starving young rabbits diminishes only the glycogen store of the muscles, leaving the liver glycogen unaffected, while Eadie [1929] claims to have shown that adrenaline has no effect on the muscle glycogen although it reduces the liver glycogen to vanishing point.

Previous experiments by one of us [Gohar, 1933] on rats have demonstrated that an injection of a convulsive dose of insulin leads to a considerable diminution of the adrenaline content of the suprarenal glands. Experiments by other workers (for literature see Gohar [1933])

suggest that an increased secretion of adrenaline is evoked by insulin. Many authors have stated that suprarenalectomy leads to an increased sensitivity of the animal to insulin [Sundberg, 1923; Houssay and Lewis, 1923; Lewis and Magenta, 1925; Artundo, 1925; Ricci and Hilami, 1925; Houssay, 1925; Tada, 1931]. These observations suggest that the mobilization of glycogen under the action of insulin may be assisted to an unknown degree by a concurrent increase in the secretion of adrenaline. The experiments described in the present communication were performed in order to determine the part which the suprarenal glands may possibly play in the action of insulin, especially with reference to the depletion of the stores of glycogen.

Method and experimental procedure.

A comparison was made between the effect of insulin on the liver and muscle glycogen in suprarenalectomized rats and rats on which a sham operation had been performed. It was shown by Karczag, Macleod and Orr [1925] that white rats, which are kept under strictly identical conditions of feeding and caging, are suitable animals for the study of the influence of various factors upon the glycogen store and upon the distribution of glycogen between the different organs. Since the figures given by these authors seem to indicate that the percentage of liver and muscle glycogen is less susceptible to variations in females than in males, we performed our experiments exclusively on female white rats. The age difference between the animals did not exceed 7 days, and all the rats were taken from a stock which had been bred in the laboratory for many generations. As recommended by Macleod and his co-workers, the animals were starved for 40 hours before the experiments, only water being allowed. They were then kept for not less than a fortnight on a mixed diet of the following composition: 60 p.c. bread, 20 p.c. meat, 15 p.c. milk, 3 p.c. cod-liver oil, 2 p.c. salts. The meat contained some fat and was minced, slightly roasted and kept in a refrigerator until required. In order to ensure a regular and simultaneous feeding, the animals were fed three times a day, at 7 a.m., 1 p.m. and 7 p.m. The food was left in the cages for 1 hour on each occasion. On the days of the experiments the animals were deprived of all food. In confirmation of the observations of Karczag, Macleod and Orr, there were no great variations in the muscle and liver glycogen in these animals, provided they were killed the same interval of time after their last meal. This was especially the case with the muscle glycogen.

On the days of an experiment two, or in some cases four, rats were selected of exactly the same weight and left without food. On one of the pair a double suprarenalectomy was performed through lumbar incisions under C.+E. anæsthesia. A sham operation was performed on the other rat, both suprarenals being exposed but left intact. Care was taken to keep both animals under anæsthesia for the same length of time. The wound was carefully closed after the operation, which usually lasted for about 10 min. The animals invariably recovered completely from the anæsthesia within 5 min. Although every pair of rats was always treated in exactly the same manner, the operations and the estimations of glycogen in the different pairs were not always performed after the same period of fasting, and therefore the amount of glycogen, although strictly comparable in the rats belonging to a single pair, showed considerable variations in animals which had the operation on different days. This difference may also have been due in part to the considerable changes in the daily temperature and the climatic conditions. White rats are particularly suitable for the purpose of these experiments since Vincent [1924] could not demonstrate in them the presence of any accessory chromaphile bodies.

The actual experiments were performed in the following manner. Two hours after the operation equal convulsive doses (4–8 units) of insulin were injected into both rats. Usually one injection was sufficient. In a few cases this was followed by a second injection, about 1 hour after the first. When one of the pair, invariably the suprarenalectomized, became comatose it was killed with a blow on the head. The other rat was allowed to survive for exactly the same length of time after the injection of the insulin as the first rat. Immediately after killing an animal its liver and thigh muscles were rapidly removed and prepared for the estimation of glycogen. Great care was taken to keep the details of the preparation and analysis constant for all the specimens. Completely untreated rats were occasionally used as controls; they were kept under the same conditions, left to starve for the same time, and in some cases kept under anæsthesia for as long as the operated rats. This provided us with information as to the percentage of glycogen which is likely to be left in the liver and muscles before the operation.

The organs used for the estimation were rapidly weighed; 1 c.c. of 60 p.c. KOH was added for each g. of tissue, and the mixture was placed in a vigorously boiling water bath under reflux for 2 hours. After being cooled, the liquid was diluted with 2 volumes of water, treated with 4 volumes of 96 p.c. alcohol and well stirred. The precipitate was allowed

to settle and then collected on a filter paper, washed once with a mixture of 1 volume of 15 p.c. KOH with 2 volumes of 96 p.c. alcohol, and afterwards washed twice with 66 p.c. alcohol. The precipitate was boiled in water, allowed to cool, neutralized with dilute HCl and filtered. After the filter paper had been washed several times with small amounts of water, the filtrate was made to a definite volume, and the glycogen concentration in the solution was determined. In preliminary experiments the glycogen determinations were carried out according to the usual method, *i.e.* hydrolysis in 2·2 p.c. HCl for 3 hours in a boiling water bath, followed by determination of the sugar by one of the recognized methods. The Hagedorn and Jensen method was used in most cases. It was soon found, however, that when the glycogen concentration is very small this procedure fails to give satisfactory results. In our experiments the amount of glycogen in animals treated with insulin or adrenaline was invariably very small. In the amounts of tissue used for analysis it was frequently only a few mg., and the solution used for the determination could not be conveniently concentrated so as to give satisfactory results with sugar-reducing methods.

Several investigators [Nerking, 1901; Kerley, 1930] have pointed out that the hydrolysis of glycogen does not give a quantitative yield of glucose. We were able to confirm this observation on a sample of pure glycogen in concentrations of the order obtained in our preliminary experiments on the liver and muscles of insulin-treated rats. The percentage loss of glucose during hydrolysis varied with the strength of the acid, the length of hydrolysis, and even with the concentration of glycogen, becoming less as the concentration increased. This introduced a factor which could obviously not be controlled. We therefore decided to use the polarimetric method of glycogen determination, which has been claimed to be more exact than sugar-reducing methods for low concentrations of glycogen.

Harden and Young [1902] have shown that, in low concentrations of glycogen where the opalescence does not interfere with the rotation, a fairly constant specific rotary power can be obtained, even for specimens of glycogen from different sources. Pflüger [1906] also recommended the direct polarimetric determination of glycogen. Using Hilger's polarimeter, and solutions of pure glycogen in concentrations similar to those obtained in our preliminary experiments, we obtained completely satisfactory results. The specific rotary power of pure animal glycogen was taken as $[\alpha]_D\ 20° = 200$ or $[\alpha]_{Hg}\ 20° = 236{\cdot}2$. The accuracy of this method diminishes with increase in the glycogen concentration, owing to opal-

escence. Below 0·4 p.c. the method was found to be accurate. Since the concentrations of glycogen in our experiments were below this figure, we adopted the polarimetric method for our work in preference to the sugar-reducing method.

Experimental results.

A comparison between the percentage of glycogen in the liver and muscles of suprarenalectomized and sham-operated rats, after the injection of convulsive doses of insulin, is given in Table I. These results and many others which were exactly similar show that in all cases the percentage of muscle glycogen in the suprarenalectomized rats injected with insulin is distinctly higher than in sham-operated rats which were treated

Table I.

Weight of each rat of the pair	Percentage of glycogen			
	Suprarenalectomized		Sham-operated	
g.	Liver	Muscle	Liver	Muscle
195	0·00*	0·27	0·00*	0·17
220	,,	0·32	,,	0·20
270	,,	0·07	,,	0·03
240	,,	0·08	,,	0·05
240	,,	0·15	,,	0·06
225	,,	0·17	,,	0·12
225	,,	0·28	,,	0·17
200	,,	0·27	,,	0·16
200	,,	0·09	,,	0·04

* Figures below 0·02 are given as zero.

in a similar manner. The liver, on the other hand, is almost completely depleted of glycogen in both cases. At first sight these results appear to show that, while the mobilization of the glycogen of the muscles is influenced by the suprarenal glands, the glycogenolysis in the liver is directly brought about through the influence of insulin and that it is independent of the discharge of adrenaline. Further observations, however, show that this is not the case.

The depletion of the liver glycogen is not due to starvation. This is shown by the figures in Table II, where a comparison is made between the percentage of liver glycogen in suprarenalectomized rats and sham-operated animals which were injected with insulin with that of livers of animals which were either simply starved or else starved and then anæsthetized for the same length of time as the operated animals. While the liver glycogen of the insulin-injected rats was reduced to vanishing point, the livers of the starved non-anæsthetized rats contained a considerable amount. The administration of the anæsthesia and the actual

operation could also not explain the almost complete disappearance of glycogen from the liver, as was shown by comparing the glycogen content of non-anæsthetized and non-operated animals with that of rats which were anæsthetized and operated upon but not injected with insulin. For this purpose a number of rats were subjected to A.C.E. anæsthesia for 10 min. each; while they were under the anæsthetic a sham suprarenalectomy was performed. The rats were then left for about 5 hours. Other rats of exactly the same weight were used as controls. The liver glycogen was found to be reduced by 40–50 p.c. in the anæsthetized rats. In no case was the depletion greater than this. These results make it evident that, although the anæsthesia and the operation led to a considerable diminution of the glycogen store of the liver, this could not explain the

TABLE II.

Weight of each of the four rats	Percentage of glycogen							
	Control rats		Suprarenalectomized rats		Sham-operated rats		Rats injected with adrenaline	
g.	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
195	0·73	0·29	0·00*	0·27	0·00*	0·17	0·00*	0·04
240	0·78	0·17	„	0·15	„	0·06	„	0·05
200	0·37	0·12	„	0·11	„	0·04	„	0·03
198	1·20	0·21	„	0·25	„	0·16	„	0·07
	Mean	0·197		0·195		0·107		0·047

* Figures below 0·02 are given as zero.

almost complete depletion of it observed after the injection of insulin. As regards the muscle glycogen, it was found to be unaffected by the length of the anæsthesia employed and the operation performed.

It can be seen from Table II that it is not only the injection of convulsive doses of insulin which leads to a complete exhaustion of glycogen from the liver but that adrenaline produces the same effect. One of a pair of rats of exactly equal weight was injected with 0·05–0·08 mg. of adrenaline, a dose of the order experimentally found to be normally present in the two suprarenal glands of similarly kept rats; the other rat served as a control. The rats were killed simultaneously 2 or 3 hours after the injection, and their livers and muscles were analysed for glycogen. The degree of depletion is striking. There is thus no reason to consider that the adrenaline secretion evoked by insulin, or by the after-effects of its administration, is without influence upon the mobilization of the liver glycogen. However, since insulin leads to as complete a depletion of the liver glycogen store even in suprarenalectomized animals, it must be concluded that the glycogenolysis in the liver is determined on the one

hand by insulin, or by the symptoms following its administration, and on the other hand by the increased secretion of adrenaline which it causes.

In the case of the muscle glycogen the results differ to a considerable extent. Insulin seems to play no direct rôle in the mobilization of the glycogen. This is shown by comparison of the percentages of muscle glycogen in the first two parts of Table II, which show that in the suprarenalectomized animals injected with insulin the amount of glycogen left in the muscles at the end of the experiments is approximately the same as in the untreated controls. The mean percentage for the suprarenalectomized rats in the four experiments is given in Table II as 0·195 p.c., and for the untreated controls as 0·197 p.c. The figures given in the table were taken from four representative experiments. A great number of other experiments not reproduced in Table II gave exactly similar results. The four in the table were selected from those which showed the greatest difference in the absolute amount of glycogen present in the muscles and liver of the control animals. Four rats of exactly the same weight were used for each experiment. A comparison of the second and third part of Table II shows that while convulsive doses of insulin fail to cause a mobilization of glycogen from the muscles in the absence of the suprarenal glands, in sham-operated animals the mobilization of the muscle glycogen is considerable. In the four experiments given in Table II the average percentage of muscle glycogen in the suprarenalectomized rats was 0·195 p.c., and in the sham-operated ones 0·107, an average diminution of about 45 p.c. Other experiments showed the same order of diminution.

These results, together with those given in Table I, show that the mobilization of the muscle glycogen caused by insulin is not brought about directly by the insulin, but through the increase in the adrenaline discharge which it produces. Further evidence in favour of this conclusion is provided by the comparison of the time which elapses between the injection of insulin and the onset of convulsions in the suprarenalectomized rats and in the sham-operated rats. Without a single exception the convulsions invariably started in sham-operated rats from 45 to 90 min. later than in the others. In one case the insulin used happened to be a less potent preparation than usual and a big dose had to be injected. The suprarenalectomized animals had convulsions and died about 15 hours after the injection, but the sham-operated rats did not even have convulsions. The general condition of the suprarenalectomized rats always aggravated more rapidly, coma developed earlier, and they died sooner than the sham-operated rats. This observation, together with the fact that the glycogen content of the muscles of the suprarenalecto-

mized rats is always higher than that of the sham-operated animals, indicates an earlier lowering of the blood sugar level in the former, owing to the diminution of the available source of glycogen in the absence of the suprarenal gland.

It is evident that the convulsions were due to hypoglycæmia, and not to the operative procedure, because of their typical nature and also because injections of glucose caused a rapid relief. Those animals which were not killed and which did not die after the injection of insulin were found to be in good condition many weeks after the operation.

It might be thought that, since the suprarenalectomized and the sham-operated rats were always killed the same time after injection of insulin, and since the suprarenalectomized animals had convulsions for not less than one hour longer than the sham-operated rats, the former would have shown a greater diminution in the glycogen content of their muscles. However, this is not the case, indicating that the diminution of the muscle glycogen content could not possibly be explained by the difference in degree of muscular activity. The muscular activity seemed to be in any case insufficient to produce an appreciable change in the glycogen store of the muscle, as shown by a comparison of the first and second parts of Table II.

Conclusions.

1. Injection of convulsive doses of insulin into rats, which are kept under strictly controlled conditions, is followed by an almost complete depletion of the liver glycogen and a considerable diminution of the muscle glycogen.

2. In suprarenalectomized animals, insulin leads to a similar diminution in the liver glycogen but apparently has no effect on the muscle glycogen.

3. Injection of adrenaline depletes the liver of its glycogen and considerably diminishes the glycogen in the muscles.

4. It is suggested that the depletion of the liver glycogen is determined by the synergetic action of insulin and adrenaline, either of them alone giving the same result as both together, while the depletion of the muscle glycogen is brought about by the insulin only indirectly through an increased activity of the suprarenal glands.

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THE EFFECT OF SPLANCHNOTOMY AND OF PHLORRHIZIN ON DECEREBRATION HYPERGLYCÆMIA[1].

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In discussing the nature of the nervous disturbance which is responsible for the development of hyperglycæmia following pontine decerebration in rabbits, Donhoffer and Macleod [1932] did not have available the results of experiments performed on animals in which the splanchnic nerves had been cut some days previous to decerebration. The present paper contains such results. It also records those obtained when the rabbits received large doses of phlorrhizin prior and subsequent to decerebration. This type of experiment was undertaken for the following reason. It was shown by Donhoffer and Macleod that hyperglycæmia which followed pontine decerebration in previously fasted rabbits was comparable in degree with that observed by Cori and Cori [1928] to be produced when 2 g. of glucose per kg. an hour were given by continuous intravenous injection into amytalized rabbits. Since this amount exceeds by many times that which could be accounted for by the glycogen which disappears from liver and muscles following pontine decerebration, it was suggested that the process of gluconeogenesis in the liver had become excessively active. The only alternative explanation for the accumulation of extra sugar in the blood is that the utilization of this substance in the tissues has become lessened. It was found, however, that the oxygen consumption, as well as the respiratory quotient, was not demonstrably influenced by the decerebration, indicating that the oxidation of carbohydrate had not become depressed. It may be noted here in passing that the stability of the respiratory quotient would also seem to indicate that gluconeogenesis had not become increased. If the oxidation of carbo-

[1] This investigation was carried out at the suggestion of Prof. J. J. R. Macleod.

[2] Carnegie Trust Scholar.

hydrate is not depressed, a lower rate of usage of glucose in the tissues might depend on a raising of the threshold at which glucose passes into them from the blood. A disturbance of this nature might also be responsible for the hyperglycæmia caused by adrenaline injections into fasting animals.

Even if the rate of sugar production by the liver were undisturbed, a small raising of the threshold of migration of glucose from blood to tissues could cause a very decided increase in the blood sugar level. It is believed that the primary cause of the glycosuria which follows phlorrhizin injections is a lowering of the sugar threshold in the kidneys, and there is not wanting some evidence that this drug also causes sugar to appear in such secretions as the bile, pancreatic juice, etc. [R. G. Pearce, 1916]. If this be the case then phlorrhizin may also lower the threshold for sugar in the muscles, and it seemed of interest to see what effect it would have on decerebration hyperglycæmia. Since, however, the lowering of the renal threshold is the most outstanding effect of the drug, as judged by comparison of the percentages of glucose in the arterial and venous blood [Nash, 1922] of the kidneys, it was necessary in certain of the present experiments to remove the kidneys by ligation of the renal vessels. The experimental procedure and the chemical methods employed in this investigation have been essentially the same as those used by Donhoffer and Macleod.

Results.

(1) *The effect of splanchnotomy.*

Splanchnotomy was performed 1–8 days prior to the decerebration experiment, the animals being anæsthetized by amytal. They were then put on a normal mixed diet, the last meal being given about 18 hours before the decerebration experiment, except in Exps. 35 and 36, when they were only fasted overnight.

A total of eight experiments were carried through, three of them on animals decerebrated through the middle of region III, three through the posterior part of the same region, one through region IV, and one between regions II and III. The results are given in Table I. In two out of the pontine decerebration experiments (viz. Nos. 53 and 54) the blood sugar failed to rise significantly, the last blood sugar value of Exp. 54 being high because it was taken from the heart when the rabbit was in a dying condition. In the third experiment of this group (viz. No. 36) the blood sugar rose from 120 mg. per 100 c.c. blood to 215 within three hours, which is much less than that usually observed.

TABLE I. The effect of pontine decerebration on splanchnotomized rabbits.

Exp. No.	Region of decerebration	Blood sugar at hourly intervals in mg. per 100 c.c. blood							Glycogen p.c. in			
									Liver		Muscle	
		Normal	1	2	3	4	5	6	Before	After	Before	After
F 36	3	120	135	180	215	215	205	—	0·74	0·33	0·19	0·22
53	3	115	120	125	130	125	120	145	1·22	0·73	0·61	0·52
54	3	120	125	140	155	250 H	—	—	1·45	0·47	—	0·59
21	3 Post.	85	160	160	150	—	—	—	0·91	nil	0·34	0·31
F 35	3 Post.	150	175	150	150	100	—	—	0·84	0·01	0·25	0·28
55	3 Slightly post.	125	130	135	130	120	140	125	3·27	1·60	0·76	0·16
31	3 Ant.	120	120	120	120	130	105	—	—	—	—	—
20	4	115	132	172	—	215	—	—	2·43	0·49	0·52	0·51

F = rabbit fasted overnight.
H = blood sample withdrawn from heart.

In the three experiments (viz. Nos. 21, 35 and 55), where the lesion was made through the posterior part of the pons, it is probable, though not certain, that the diabetic centre was touched at some point along the line of decerebration. In only one of these three experiments (viz. No. 21) was there a small degree of hyperglycæmia, the blood sugar rising from 85 mg. per 100 c.c. blood to 160. In all these experiments save one (No. 55) the liver glycogen percentage fell as usual, and in all there was very little, if any, change in that of the muscles.

The results of the experiment (No. 31), in which the decerebration was through the anterior portion of the pons, are doubtful, since it occasionally happens that under these conditions the blood sugar level is not disturbed.

The results, as a whole, clearly show that splanchnotomy prevents the development of hyperglycæmia following pontine decerebration, and they indicate that the muscle glycogen is not diminished to the same extent as when the splanchnic nerves are intact.

(2) *The effect of phlorrhizin.*

Rabbits which had been fed on an ordinary mixed diet were fasted overnight or for longer periods (18–36 hours). Twenty to thirty minutes prior to decerebration under amytal anæsthesia 0·75 g. phlorrhizin was given subcutaneously and, where indicated in the table (Table II), the

TABLE II. Effect of phlorrhizin on decerebration hyperglycæmia.

Exp. No.	Region of decerebration	Initial dose of phlorrhizin g.	Blood sugar at hourly intervals in mg. per 100 c.c. blood								Liver glycogen p.c.		Glycosuria	Remarks
			Normal	1	2	3	4	5	6	7	Before	After		
F 45	3	0·75	130	115	*95	95	135	*175	160 H	175	—	—	present	Kidney vessels not tied
F 46	3	,,	115	130	130	140	*140	155	215	—	5·00	1·25	,,	,,
38	3	,,	60	150	210	250	—	210	190	—	—	1·90	—	Kidney vessels tied
F 48	3	,,	130	270	270	295	310	*330	320	—	1·95	nil	—	,,
F 49	3	,,	115	185	170	*200	290	*—	360	—	3·94	nil	—	,,
34	2	,,	110	110	110	105	105	85	—	—	—	nil	—	,,
44	2	,,	120	190	165	*140	165	145	—	—	—	—	present	Kidney vessels not tied
37	Ant. 3	0·5	110	120	165	170	205	220	—	—	—	1·75	—	Kidney vessels tied
40	Post. 3	,,	180	200	245	270	295	310	—	—	—	—	present	Kidney vessels not tied
47	4	0·75	175	260	*205	185	200	220	*215	—	11·43	6·97	,,	,,

* Subsequent subcutaneous injections of 0·25 g. phlorrhizin.
F = Rabbit fasted overnight.
H = Blood sample withdrawn from heart.

kidney vessels were ligated. A sample of liver was also removed for estimation of glycogen by the Pflüger method, and a sample of blood withdrawn for estimation of the blood sugar. In Exps. 37 and 40 only 0·5 g. phlorrhizin was given.

Asterisks in the table mark subsequent doses of 0·25 g.

After decerebration blood samples were withdrawn from the ear at intervals of one hour. The experiment ended with the removal of a piece of liver for glycogen estimation and, when the kidney vessels were not tied, of a sample of urine to test for the presence of sugar by Benedict's method.

In five of the experiments the lesion was definitely through the centre of region III. In two of them (viz. Nos. 45 and 46) the kidneys were not tied off and no significant hyperglycæmia developed. In the remaining three experiments (Nos. 38, 48 and 49), where the kidneys were ligated, a definite and prolonged rise in blood sugar percentage followed decerebration.

Where decerebration was performed at other levels the results were not so orderly.

In Exp. 37 the line of decerebration was at the anterior border of the pons, so that it is impossible to say whether it was the phlorrhizin or the anterior position of the lesion which was responsible for there being only a moderate rise in the blood sugar level. In Exp. 40, in which the decerebration was through the posterior portion of the pons and the kidney vessels were intact, hyperglycæmia became developed, possibly because only a small amount of phlorrhizin was injected at the beginning of the experiment. The results of Exps. 34 and 44, in which decerebration was distinctly anterior to the pons, the blood sugar level did not change except temporarily, whether or not the renal vessels were tied, showing that this operation and the administration of phlorrhizin do not in themselves have an effect on the blood sugar level.

Conclusions.

1. Pontine decerebration in fasted rabbits in which the splanchnic nerves have been cut several days previously is not followed by an increase in the blood sugar percentage.

2. Injection of large doses of phlorrhizin does not prevent the development of decerebration hyperglycæmia when the renal vessels are ligated, but does so when these are intact. This result supports the view that phlorrhizin acts directly on the kidneys.

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THE INFLUENCE OF VAGAL STIMULATION UPON CONDUCTION THROUGH THE BRANCHES OF THE A.-V. BUNDLE IN THE DOG.

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It is a common observation that when the vagus nerves are stimulated an impulse passing from the auricle to ventricle travels with difficulty; it either suffers delay or is partially or completely blocked, according to the degree of stimulation employed. The disturbed impulses usually reach the ventricle in a perfectly normal manner, giving rise to normal ventricular complexes. On occasions, however, they travel abnormally, reaching one ventricle before the other, so that abnormal complexes occur during the stimulation. These take place both when the block between the auricle and ventricle is complete [Einthoven, 1906; Kahn, 1909; Kraus and Nicolai, 1910] and also when it is partial; but recorded examples of the latter are few. Hering [1909] reported aberrant ventricular complexes in a dog during vagal stimulation, the ventricular beat being preceded by an auricular beat. Kraus and Nicolai [1910] stimulated the right auricle during vagal inhibition and obtained aberrant ventricular complexes. Rothberger and Winterberg [1911] have published curves in which each aberrant ventricular complex was preceded by an auricular wave. In these experiments, however, the vagal effect was complicated by a simultaneous accelerator stimulation. Einthoven and Wieringa [1914] published curves from dogs during morphine poisoning. Certain of these during partial heart block show aberrant ventricular complexes to supraventricular stimuli. This rhythm was abolished by atropinization. In 1915 Wilson reported a clinical case in which a perfectly normal cardiac rhythm could be changed by deep breathing or vagal pressure into one in which the pacemaker was situated

[1] Working on behalf of the Medical Research Council.

high up in the A.-V. node and the ventricular complexes were aberrant. When this rhythm occurred spontaneously it could be abolished by atropine. In all these instances the impulse giving rise to the aberrant beat arose either in the auricle or high up in the A.-V. node. It travelled presumably through the main branch of the bundle and the bundle branches. The explanation which was offered by Einthoven and Wieringa [1914] for such beats was that the vagus nerves influence conduction not only in the A.-V. node and bundle, but also in the bundle branches, and that the group of fibres supplying one of the bundle branches reacted more strongly so that conduction in this branch was relatively more disturbed than in the other, and aberrant ventricular complexes thus arose from supraventricular stimuli.

As far as concerns those observations in which complete block is present, the idioventricular rhythm may arise high in the A.-V. bundle and the impulse be delayed in one of the bundle branches by vagal stimulation. It may equally well be that the idioventricular rhythmic centre is situated in one of the bundle branches. Both conditions would give rise to abnormal ventricular complexes.

These observations suggest that vagal stimulation influences branch conduction. Lewis [1914] has shown, however, that the chief action of the vagus is at the A.-V. node or its immediate neighbourhood. It seemed desirable therefore to find out whether or not vagal stimulation delays conduction in the bundle branches.

The method we have used depends upon the following considerations.

Lewis and Rothschild [1914] have shown that when the left ventricle of a dog is stimulated there are two paths open for the impulse to take as it travels to the right ventricle. It can pass entirely through muscle or it can reach the Purkinje tissue lining the left ventricular cavity, pass up the left and down the right bundle branch, and spread through the Purkinje tissue lining the right ventricle and so reach the muscle. Owing to the slow rate of conduction through muscle, compared to the high rate through Purkinje tissue, they found that the impulse must travel to the distant parts by way of the Purkinje system and the bundle branches, as it reaches these regions before it could do so if it travelled by muscle tracts only. We can therefore liberate an impulse which in its course will travel through the bundle branches, and by studying its rate of propagation both normally and during vagal stimulation, determine whether such stimulation has any influence.

Aberrant ventricular complexes are due to the delayed arrival of the excitation wave at the termination of one or other bundle branch.

Lewis [1916] showed that in the right bundle branch block the first part of the Q.R.S. complex is due to the spread through the left ventricle; in left bundle branch block, to the spread through the right ventricle. If the pure right ventricular effects and pure left ventricular effects were added algebraically in their proper time relations, a perfectly normal Q.R.S. complex was obtained. Wilson and Herman [1921] using the data of Lewis have determined the effect of shifting the relative position of the dextro- and lævocardiogram upon the composite wave, and have shown that a shift of 0·005 sec. produces curves which are transitional between normal and bundle branch block curves; they are aberrant in form. From these observations it appears evident that the delay in conduction through one bundle branch must be at least of the order of 0·005 sec. greater than that through the other if aberrant beats are to occur. Even if it is assumed that the disturbance in conduction produced by vagal stimulation is confined to one bundle branch, the delay, namely 0·005 sec., should be appreciated by the method we have employed. The more likely result of such stimulation, however, is a delay in both branches, with that in one branch greater than that in the other. In these conditions a considerable delay in the passage through both bundle branches would be expected, and would be clearly seen in the observations which we have made.

Method.

Dogs of about 10 kg. were used, and were anæsthetized with morphia followed by paraldehyde and, when necessary, a sufficiency of ether. Under artificial respiration the thorax was opened and the heart exposed. The pericardium was cut open and sutured to the chest wall, forming a cradle in which the heart freely beat. A radiant heater suitably placed maintained constant body temperature. The vagus nerves were exposed in the neck and stimulated by means of a faradizing current; they were usually cut.

The arrival of the excitation wave at different points on the ventricular surface was determined by placing an electrode upon the muscle and connecting it to a string galvanometer. The electrode consisted of an outer silver tube about 1/8 in. diameter, through the centre of which a fine insulated silver wire passed. The outer tube and inner wire formed the two leads to the galvanometer. The inner wire projected about 1 mm. beyond the outer tube and when in position upon the heart penetrated the muscle. This form of electrode was employed as it gives a very similar electrical response wherever it was placed upon the ventricular

muscle. When it was connected to the galvanometer so that activity beneath the inner wire caused an upward deflection, the record was diphasic, a small slowing rising upward deflection being followed abruptly by a large rapidly falling downward deflection. The downward deflection was therefore associated with activity beneath the outer silver tube. The beginning of activity beneath the small central wire was not to be readily appreciated so we have used the beginning of the large deflection for our measurements. With the record of this deflection, a lead II electrocardiogram was recorded simultaneously. This serves not only as an index of the constancy of the rhythm but allows the arrival of the excitation wave on the ventricular surface to be correlated with a "standard" position in lead II.

Lewis and Rothschild [1914] in their observations employed two separate electrodes, one upon the muscle, the other upon indifferent tissue nearby. We have compared the time measurements as obtained by this method with those by the silver tube electrode and have been unable to note any difference. We have preferred to use the tube electrode, as it eliminates in large measure the electrical effects due to activation of muscle other than that immediately beneath the contacts, and gives curves which are similar at all positions upon the ventricle. It is also less liable to disturbance from the escape of current used to stimulate the vagus.

The records were taken upon a moving paper camera, and measured by means of a Cambridge type record measurer, the error of which amounts to about 0·003 sec.

Left ventricular extrasystoles.

The spread of the excitation wave over the surface of the right ventricle.

Lewis and Rothschild [1914] have offered evidence that when an impulse is liberated in the left ventricle, it passes to the distant portions of the contralateral ventricle by way of the bundle branches. The activation of the opposite ventricle is, however, a race between impulses arriving by the bundle branches and those arriving by muscle spread. It is so important to the interpretation of these present observations that there should be no doubt as to the precise path taken by the excitation wave in such circumstances that we have examined the matter in some detail.

In our experiments we have determined the order of activation of the right ventricle during a series of normal beats and then during a

series of extrasystolic beats forced from the left ventricle. Left ventricular extrasystoles were chosen as the right ventricular surface is easy of access experimentally, and the order of activation in this ventricle is well defined owing to the distribution of the right bundle branch of the A.-V. bundle.

The extrasystoles have been obtained by driving the left ventricle by means of rhythmic shocks passed through fishhook electrodes embedded in the musculature, the position of which has been varied in the different experiments. The rhythmic rate used has been slightly above that of the natural heart rate. The spread of the excitation wave over the surface of the right ventricle has been determined by placing the recording electrode at a series of different positions and finding the order, in time, of the activation of these points. This has been done for the normal rhythm and immediately afterwards for the left ventricular extrasystolic rhythm. The time of arrival of the excitation wave has been measured with reference to a standard point in lead II in each instance. With the normal rhythm, the beginning of the R. wave, and with the extrasystolic rhythm, the beginning of the main ventricular deflection have been used.

The results of such experiments are shown in Table I. In this table the excitation wave is considered to reach the earliest activated position at zero time, and the delay in its arrival at the other positions is calculated with reference to this first position. The order of activation is also tabulated. The actual positions chosen and the points of stimulation are indicated in the diagrams (Table I). The measurements show that as far as the normal rhythm is concerned the right central portion is activated first, then the right middle, and finally the portion of the right ventricle close to the A.-V. groove. These results are in agreement with those obtained by Lewis and Rothschild [1914], and are explained by the fact that the right bundle branch through which the normal impulse reaches the ventricle does not break up into the Purkinje network till it reaches a position close to the right central region. The delay in the arrival at points distant from this is due to the time required for the spread through the Purkinje network. It is also to be noted that the actual time values correspond very closely with those observed by Lewis and Rothschild [1914]. When the extrasystolic measurements are considered, it is evident that while the order of activation in the main corresponds with that of the normal rhythm, there are many discrepancies. Although certain of these lie within the limits of experimental error it would be unjustified without further evidence to con-

TABLE I. Spread of excitation wave over right ventricular surface during (*a*) normal rhythm, (*b*) a series of left ventricular extrasystoles.

Position	Normal rhythm Time	Normal rhythm Order	Left ventricular extrasystole Time	Left ventricular extrasystole Order
Exp. 3.6.22. Vagus nerves intact.				
1	0·0000	1	0·0000	1
2	0·0059	2	0·0047	3
3	0·0077	3	0·0036	2
4	0·0084	4	0·0051	4
5	0·0145	5	0·0145	7
6	0·0155	6	0·0138	6
7	0·0161	7	0·0126	5
Heart rate per min.	194		218	
Exp. 3.6.28. Vagus nerves cut.				
1	0·0000	1	0·0000	1
2	0·0081	2	0·0015	2
3	0·0123	3	0·0132	5
4	0·0151	4	0·0102	3
5	0·0162	5	0·0112	4
6	0·0168	6	0·0139	6
7	0·0218	7	0·0173	7
8	0·0246	8	0·0222	8
Heart rate per min.	200		230	
Exp. 3.7.11. Vagus nerves cut.				
1	0·0000	1	0·0000	1
2	0·0081	2	0·0077	3
3	0·0095	3	0·0063	2
4	0·0190	4	0·0154	5
5	0·0206	5	0·0116	4
6	0·0217	6	0·0164	6
Heart rate per min.	187		230	
Exp. 3.7.18. Vagus nerves cut.				
1	0·0000	1	0·0155	4
2	0·0011	2	0·0070	2
3	0·0050	3	0·0000	1
4	0·0082	4	0·0153	3
Heart rate per min.	175		225	
Exp. 3.7.28. Vagus nerves cut.				
1	0·0000	1	0·0000	1
2	0·0079	2	0·0067	4
3	0·0090	3	0·0013	2
4	0·0095	4	0·0027	3
5	0·0131	5	0·0110	6
6	0·0142	6	0·0090	5
Heart rate per min.	180		200	
Exp. 3.8.4. Vagus nerves cut.				
1	0·0000	1	0·0054	3
2	0·0020	2	0·0000	1
3	0·0111	3	0·0184	5
4	0·0113	4	0·0168	4
5	0·0119	5	0·0040	2
6	0·0228	6	0·0208	6
Heart rate per min.	185		210	
Exp. 3.10.13. Vagus nerves cut.				
1	0·0000	1	0·0124	4
2	0·0067	2	0·0000	1
3	0·0117	3	0·0121	3
4	0·0129	4	0·0243	6
5	0·0142	5	0·0249	5
6	0·0142	6	0·0055	2
Heart rate per min.	200		220	

Exp. 3.6.22. Exp. 3.6.28. Exp. 3.7.11. Exp. 3.7.18. Exp. 3.7.28. Exp. 3.8.4. Exp. 3.10.13.

Diagrams illustrating the positions at which the recording electrode was placed and the point stimulated (*S*).

clude that the activation of all points in the right ventricle was from impulses which passed down the right bundle branch. If the position of the point of stimulation is such that the central portion of the right ventricle is reached early by muscle spread, the activation of the remainder will follow the normal sequence, for the impulse will spread from this point by the Purkinje tissue as it does normally. To try to obviate this we have stimulated the left ventricle from different positions, namely dorsal and ventral surfaces, and basal and apical regions. In two experiments using the electrodes already described [Dale and Drury, 1932] we stimulated the left ventricle endocardially in the apical region. In none of the experiments was the order of activation identical with that of the normal rhythm. In consequence of this we proceeded to cut the right bundle branch and to compare the time of activation of chosen points in the right ventricle before and after such a section. In these experiments the arrival of the excitation wave was recorded at certain positions during a series of left ventricular extrasystoles, before and after the right bundle branch was cut. The success of this section was shown by the appearance of the typical electrocardiogram in lead II during the normal rhythm, and in Exps. 3.7.18, 3.7.28, and 3.8.4 by the additional observation that, with a normal rhythm, the activation of all the points on the right ventricle was considerably delayed, when compared with measurements obtained before section. The results are tabulated in Table II. The measurements show clearly that we have not been able to find a point of stimulation from which all the positions on the right ventricle are activated by impulses arriving by the right bundle branch. The arrival of the excitation wave at certain positions is definitely delayed by the section: Exp. 3.6.28, positions 1 and 6; Exp. 3.7.18, position 2; Exp. 3.7.28 (*a*), positions 2 and 3; Exp. 3.7.28 (*b*), positions 2, 3, 4, 5 and 6; Exp. 3.8.4, position 1; Exp. 3.10.13, position 5. The delay in other positions is less certain: Exp. 3.6.28, position 2; Exp. 3.7.28 (*a*), positions 1, 4 and 6. But there is no delay in the arrival at one or more points in every experiment: Exp. 3.6.28, position 5; Exp. 3.7.18, positions 1, 3 and 4; Exp. 3.7.28 (*a*), position 5; Exp. 3.7.28 (*b*), position 1; Exp. 3.8.4, positions 2, 3, 4, 5 and 6; Exp. 3.10.13, positions 1, 2, 3, 4 and 6. It can only be concluded that these last positions have been activated by impulses which have travelled through muscle and not through the right bundle branch. As far as our observations go, we feel that it is unlikely that the right ventricle will be activated solely by impulses arriving by the bundle branch, whatever position on the epicardium or endocardium of the left ventricle is chosen

as the point of stimulation. If precise information is desired as to which positions are activated by impulses arriving by the bundle branch, then the branch must be cut in every experiment.

TABLE II. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles (*a*) before and (*b*) after section of right bundle branch.

	Position*	Normal	Branch cut
Exp. 3.6.28.	1	0·0205	0·0403
	2	0·0134	0·0175
	5	0·0304	0·0297
	6	0·0396	0·0594
	Heart rate 230 per min. Vagi cut.		
Exp. 3.7.18.	1	0·0294	0·0261
	2	0·0209	0·0309
	3	0·0139	0·0136
	4	0·0292	0·0265
	Heart rate 225 per min. Vagi cut.		
Exp. 3.7.28.	1	0·0312	0·0261
	2	0·0280	0·0397
	3	0·0226	0·0361
	4	0·0241	0·0293
	5	0·0323	0·0286
	6	0·0293	0·0336
	Heart rate 200 per min. Vagi cut. Stimulation at vortex of left ventricle. Epicardium.		
	1	0·0211	0·0206
	2	0·0250	0·0427
	3	0·0247	0·0399
	4	0·0264	0·0334
	5	0·0270	0·0354
	6	0·0289	0·0345
	Heart rate 200 per min. Vagi cut. Stimulation at vortex of left ventricle. Endocardium.		
Exp. 3.8.4.	1	0·0156	0·0251
	2	0·0084	0·0095
	3	0·0305	0·0293
	4	0·0304	0·0332
	5	0·0165	0·0125
	6	0·0405	0·0388
	Heart rate 190 per min. Vagi cut. Stimulation at vortex of left ventricle. Endocardium.		
Exp. 3.10.13.	1	0·0124	0·0146
	2	0·0000	0·0000
	3	0·0121	0·0149
	4	0·0243	0·0240
	5	0·0219	0·0290
	6	0·0055	0·0083
	Heart rate 220 per min. Vagi cut. Stimulation at vortex of left ventricle. Epicardium.		

* See diagrams at bottom of Table I.

Influence of vagal stimulation upon conduction through the bundle branches.

The observations in the preceding section show clearly that the spread from left to right ventricle is a race between impulses arriving by the right bundle branch and those arriving by muscle. Although it is true that precise information with regard to the influence of vagal stimulation upon conduction is only obtained in those experiments in which the positions activated by impulses arriving by the bundle branch have been determined, the experiments in which no such observations have been made can be usefully employed. Certain positions are apparently activated by impulses arriving by the bundle branches in every experiment, and if it is found that the measurements at all positions are unchanged, then this is presumptive evidence that the vagus nerves have no influence upon branch conduction. If in addition the measurements from the experiments in which the positions activated by branch spread are known, and show no change during the stimulation, then it may be safely concluded that conduction is undisturbed.

In these experiments the arrival of the excitation wave at a point on the surface of the right ventricle was recorded during a series of left ventricular extrasystoles. The right vagus nerve was then stimulated in the neck with a current sufficient completely to arrest the heart; extrasystoles were forced, and the arrival again recorded. The left vagus nerve was then stimulated and another record taken. When acetylcholine was employed, the dose used was enough to arrest the heart completely for 30 sec. when intravenously injected. The electrode upon the heart was then moved to a new position, and the same procedure carried out. A series of positions were tested as quickly as possible one after another, and the measurements are shown in Table III. In some experiments the right bundle branch was cut immediately after these observations had been made and the time of activation of the positions on the right ventricle measured once more. The positions activated by impulses arriving by the bundle branches were thus determined and are shown in heavy type in the table.

The measurements in all the experiments tabulated clearly show that vagal stimulation has no influence upon the spread of the excitation wave. While at some positions a decrease in the time measurement is noted, at others an increase is found. These differences are all small, and fall within the limits of experimental error. The impulses, however, which have travelled to the contralateral ventricle by the bundle

TABLE III. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles (*a*) normal and (*b*) during vagal stimulation.

	Position*	Normal	Right vagal stimulation	Left vagal stimulation
Exp. 3.6.22.	1	0·0196	0·0217	0·0237
	2	0·0313	0·0282	0·0312
	3	0·0307	0·0294	0·0317
	4	0·0263	0·0222	0·0269
	5	0·0372	0·0399	0·0353
	6	0·0385	0·0392	0·0388
	7	0·0377	0·0370	0·0333
Heart rate 215 per min. Stimulation at ventral surface of left ventricle. Vagi intact.				
Exp. 3.6.28.	1	0·0205	0·0238	0·0244
	2	0·0220	0·0207	0·0210
	3	0·0337	0·0343	0·0342
	4	0·0306	0·0306	0·0290
	5	0·0317	0·0323	0·0309
	6	0·0344	0·0338	0·0315
	7	0·0378	0·0375	0·0390
	8	0·0427	0·0420	0·0415
Heart rate 230 per min. Stimulation at ventral surface of left ventricle. Vagi cut.				
Exp. 3.7.11.	1	0·0281	0·0327	0·0308
	2	0·0358	0·0357	0·0310
	3	0·0344	0·0307	0·0355
	4	0·0435	0·0413	0·0429
	5	0·0397	0·0393	0·0405
	6	0·0445	0·0437	0·0426
Heart rate 230 per min. Stimulation at lateral surface of left ventricle. Vagi cut.				
Exp. 3.7.28.	1	0·0213	0·0231	0·0237
	2	0·0280	0·0293	0·0261
	3	0·0226	0·0219	0·0224
	4	0·0241	0·0260	0·0271
	5	0·0323	0·0308	—
	6	0·0293	0·0306	0·0302
Heart rate 200 per min. Stimulation at vortex of left ventricle, epicardial surface. Vagi cut.				
	1	0·0211	0·0217	0·0207
	2	0·0250	0·0247	0·0253
	3	0·0247	0·0229	0·0237
	4	0·0264	0·0253	0·0276
	5	0·0264	0·0249	0·0279
	6	0·0289	0·0271	0·0300
Heart rate 200 per min. Stimulation at vortex of left ventricle, endocardial surface. Vagi cut.				
			Acetylcholine	
Exp. 3.7.18.	1	0·0305	0·0342	
	2	0·0349	0·0328	
	3	0·0226	0·0243	
	4	0·0319	0·0307	
Heart rate 175 per min. Stimulation at dorsal surface of left ventricle. Vagi cut.				
			Acetylcholine	
Exp. 3.8.4.	1	0·0319	0·0325	
	2	0·0265	0·0243	
	3	0·0349	0·0335	
	4	0·0433	0·0415	
	5	0·0305	0·0334	
	6	0·0473	0·0474	
Heart rate 190 per min. Stimulation at vortex of left ventricle, endocardial surface. Vagi cut.				

* See diagrams at bottom of Table I.

branches, must have passed in addition through muscle and Purkinje tissue from the point of stimulation on the surface of the left ventricle to the recording electrode on the surface of the right ventricle. No conclusions therefore can be drawn in respect of the influence of vagal stimulation upon conduction through the bundle branches until it can be shown that such stimulation has no influence upon conduction through ventricular muscle or Purkinje tissue. Many of the positions on the contralateral ventricle, however, are activated by impulses which have travelled through muscle and, in all probability, Purkinje tissue. As these measurements are unaffected by vagal stimulation (Table III), it is reasonable to conclude that the stimulation has no influence upon conduction through these two tissues. There is, however, additional evidence. One of us [Drury, 1923], using a method which allowed the rate of conduction through ventricular muscle to be measured, was unable to find that vagal stimulation had any influence. Recently we have carried out direct observations upon the effect of vagal stimulation on conduction through ventricular muscle and Purkinje tissue combined. The method used has been to test the rate of conduction through a long stretch of right ventricular muscle. If a sufficient length of muscle is taken, owing to the high rate of conduction through Purkinje tissue compared to that through muscle, the impulse reaches the distant parts more quickly by passing inwards to reach the Purkinje tissue and outwards again through muscle, than by travelling through muscle alone [Lewis and Rothschild, 1914]. This pathway will be chosen if the distance is more than twice the thickness of the muscle wall. We have therefore stimulated the right ventricle and placed one pair of recording electrodes close to the point stimulated, and another pair at a distance away greater than twice the thickness of the muscle. In such circumstances we are unable to find that vagal stimulation has any influence upon the rate of conduction.

From these experiments it is safe to conclude therefore that vagal stimulation does not influence either conduction through ventricular muscle and Purkinje tissue or through the bundle branches. There is one position in the path travelled that we have been unable to test. We have no evidence of the influence of vagal stimulation upon the crossing of the impulse from the left to the right bundle branch. It might be argued that the vagal effect was to slow conduction in the bundle and to speed up the crossing of the impulse from the left to the right bundle branch. The fact that our measurements remain undisturbed renders such an explanation extremely unlikely, for the two effects would scarcely counterbalance one another so precisely.

During a series of left ventricular extrasystoles the ventricular complexes in lead II are unchanged by vagal stimulation; they are, in fact, superimposable in every way. This, in itself, is evidence that there has been no disturbance of conduction. Careful measurement of the extrasystolic curves from lead II before and after right bundle branch section shows that the duration of the complex is usually increased by about 0·01 sec., the Q.R.S. group being slightly widened. The activation of those positions supplied by impulses travelling by the bundle branches is delayed by about 0·01–0·02 sec. when the right bundle branch is sectioned, so that the observed increase in duration of the complex agrees very closely. In some experiments, however, we can detect no appreciable change in the curves before and after branch section. Although, therefore, the fact that vagal stimulation has no influence upon lead II may be taken as evidence that the conduction through the bundle branches is undisturbed, it is to be noted that the change produced by section of the right bundle branch is often very slight.

The region at which the impulses pass from the left to the right branch of the A.-V. bundle.

When an extrasystole is forced from the left ventricle and the excitation wave travels to the right ventricle by means of the bundle branches, it must cross at some region from the fibres which lead from the left ventricle to those which lead to the right ventricle. Experiments of the type already described give some idea of the region at which impulses can so pass.

In Exp. 3.7.28 the animal exhibited a condition which is occasionally seen. When the left ventricle was driven by rhythmic shocks at a rate slightly above that of the natural rhythm the impulse completely failed to pass to the auricle, complete retrograde heart block being present. In addition, when an auricular beat occurred at a suitable moment during the extrasystolic rhythm, it passed readily to the ventricle, and gave rise to a supraventricular response, thereby disturbing the extrasystolic rhythm. There was no indication of any degree of forward heart block. This disturbance to the extrasystolic rhythm was obviated in the experiment in question by driving the ventricle and auricle simultaneously.

When the time measurements in the experiment in question are considered, it is seen that the excitation wave arrives at the surface of the right ventricle in much the same time as in experiments in which no retrograde heart block was seen (Table III). This is true for positions

which are known to have been activated by excitation waves passing through the right bundle branch. It may therefore be concluded that the impulse liberated in the left ventricle passed through the bundle branches without any abnormal delay, and crossed below the place at which the retrograde heart block occurred.

In Exp. 3.7.11 the A.-V. bundle was cut, and complete heart block resulted. At post-mortem, the cut was found to be 2 mm. deep and 5 mm. long and was situated in the long axis of the heart, about 1·0 cm. to the left of the opening of the coronary sinus, and at the same level. The activation of positions on the right ventricular surface was uninfluenced by the section (Table IV). It is not possible to say which

TABLE IV. Time in seconds of activation of right ventricular surface relative to main ventricular deflection in lead II during a series of left ventricular extrasystoles. Influence of (*a*) section or clamp of main bundle and (*b*) section of right bundle branch.

	Position*	Normal	Main bundle destroyed	Branch cut
Exp. 3.7.11.	1	0·0368	0·0376	—
	2	0·0357	0·0311	—
	3	0·0242	0·0294	—
	4	0·0417	0·0424	—
		Heart rate 230 per min. Vagi cut.		
Exp. 3.8.4.	2	0·0319	0·0306	0·0401
		Heart rate 190 per min. Vagi cut.		
Exp. 3.10.13.	3	0·0209	0·0213	0·0290
		Heart rate 220 per min. Vagi cut.		

* See diagrams at bottom of Table I.

positions were activated by impulses passing through the right bundle branch, as this was not cut in this experiment. It is reasonable from our observations to assume that some of the positions were so activated. As all the time measurements remain undisturbed, it is evident that the passage from the left to the right bundle branch occurred below the section.

In Exps. 3.8.4. and 3.10.13 the bundle was compressed with a clamp and complete heart block produced. At post-mortem bruised areas about 0·8 cm. long and 0·3 cm. wide, roughly corresponding to the size of the blade of the clamp, were found situated about 3 and 5 mm. respectively to the left of the opening of the coronary sinus and at the same level. The times of activation of positions on the right ventricle, which were found later by right bundle branch section to have received their impulses by the right bundle branch, were undisturbed by this procedure (Table IV). It is evident also from these experiments that the impulse must have crossed from the left to the right bundle branch at a position

close to the bifurcation, as although the main pressure of the clamp is exerted at its point of application, a definite though lesser pressure must be exerted a distance away. The precise position at which an impulse can cross is not to be decided by such experiments, but it is certainly well below the A.-V. node itself.

Discussion.

Although it is possible, in some of the recorded instances, that the aberrant ventricular beats which are in apparent response to supraventricular stimuli are in reality ventricular escaped beats which coincide in time with the expected supraventricular beats, it is not easy to offer this explanation in all cases. Moreover, aberrant beats are seen when the septal branch of the left coronary artery is ligated [Kahn, 1911; Lauterbach, 1928]. We feel therefore that supraventricular stimuli are capable of giving rise to aberrant ventricular complexes under certain conditions. The simple explanation suggested by Einthoven and Wieringa [1914], namely, that the vagus nerves influence conduction in the bundle branches, and that their influence is sometimes exerted more strongly upon one branch than the other, so that the impulses reach one ventricle before the other and give rise to aberrant ventricular beats, is not supported by our observations. In fact our results show so clearly that stimulation of the vagus nerves, either by faradization in the neck or by the injection of acetylcholine, has no influence upon conduction in the bundle branches, that the explanation can no longer be accepted.

Two other possible explanations suggest themselves. The first is that the supraventricular impulses pass into right and left bundle branch tracts at a position in the bundle below which no crossing is possible, and this differentiation occurs at a region where vagal stimulation slows down conduction. If the vagal action is unequal, aberrant ventricular beats will occur. On this theory also, the impulses which pass upward through one branch will not be expected to cross to the other until they reach the region above that at which differentiation into two separate tracts occurs and where the vagus nerves slow down conduction. We are unable to find any evidence that the impulses are slowed. Moreover, we find that those impulses passing upward through the left bundle branch can cross to the right at relatively low levels. On these grounds we feel this explanation is untenable unless it is accepted that impulses travelling from the auricle to the ventricle are unable to cross at the same low levels as those which cross from branch to branch.

The second is that the junctional tissues (*i.e.* node, bundle and branches) between auricle and ventricle conduct with a decrement.

This is evidence that conduction with a decrement occurs in damaged auricular muscle [Drury, 1925; Drury and Andrus, 1924]. It also occurs during the period of recovering conduction in normal auricular muscle [Drury and Regnier, 1929]. It has not been noted in normal auricular muscle after full recovery of conduction has taken place, as the method of experiment available does not allow of either proof or disproof. Schmitt [1928] has demonstrated decremental conduction in strips of turtles' ventricular muscle poisoned with potassium chloride. Decremental conduction through the junctional tissue is strongly suggested by the work of Lewis and Master [1925] on heart block produced by vagal stimulation, asphyxia and quinidine. There is considerable evidence that decremental conduction can occur over long stretches of cardiac tissue in contradistinction to nerve where it is limited to very short stretches [Edwards and Cattell, 1927].

Conduction through a decremental area can be disturbed in two ways, the gradient of decrement may become more steep or the strength of the impulse entering the region may be weakened. Schmitt [1928] has shown if an impulse entering a region of constant decrement is weakened, it travels with greater difficulty. Vagal stimulation does not disturb conduction through the bundle branches so that it must be concluded that it does not change the gradient of decrement in this region. If, however, it increases the gradient of decrement at the higher levels, or decreases the impulse strength (*i.e.* the ability to pass through a region of disturbed conduction), then the impulse will be below normal strength when it arrives at the bundle branches. A local condition constantly present, and not connected with vagal stimulation, may steepen the gradient more in one branch than the other, so that the weakened impulse will be relatively more delayed in one branch than the other or even run to extinction, while a normal impulse would pass without difficulty[1].

In those conditions in which complete vagal heart block is present, and the idioventricular beats are abnormal, we feel that the more likely explanation is that the impulses arise in the bundle above its bifurcation and are subject to decremental conduction rather than that they arise in a bundle branch.

[1] Recent observations made by us lend support to this conception.

SUMMARY.

1. The order of activation of the right ventricular surface during a series of left ventricular extrasystoles is similar to but not identical with that found during a normal rhythm.

2. Wherever the left ventricle is stimulated some positions on the right ventricle are activated by impulses which travel through muscle, others by those which travel through the bundle branches.

3. Vagal stimulation has no influence upon the spread of these impulses, whether they are travelling through muscle or through the bundle branches. It is concluded therefore that vagal stimulation does not disturb conduction in the branches of the A.-V. bundle.

4. The relation of this finding to the aberrant ventricular beats seen during vagal stimulation is discussed.

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THE BIOLOGICAL SIGNIFICANCE OF THE LINKAGES IN ADENOSINE TRIPHOSPHORIC ACID.

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For some years before 1929 interest had been aroused in the adenyl group of compounds by the recognition of the important part played by them in the glycolytic processes of muscular activity. Since then, additional significance has been attached to them because of the demonstration by Drury and Szent-Györgi [1929] of certain characteristic biological actions. After having studied the activity of simple saline extracts of cardiac muscle, they isolated from the trichloroacetic acid extract of bullock's heart a crystalline substance which their analysis showed to be adenylic acid. This acid was undoubtedly derived from the adenosine triphosphoric acid (a.t.p.) present in the original extract.

They found that adenylic acid produced a typical heart block in the guinea-pig, dilatation of the coronary arteries, lowering of blood-pressure in the dog, and diminution in the amplitude of contraction of isolated strips of intestine.

Lindner and Rigler [1930] found that extracts of the sinus, node of Tawara, and bundle of His from the calf's heart, prepared in a manner not specified, stimulated an hypodynamically acting frog's heart. They attempted to fractionate their extracts, and eventually prepared a substance which dilated the coronary arteries in the mammalian heart, and had no effect on the frog's heart. This substance they found to contain a pentose and a purine base.

Work of this nature, including that of Rigler and Schaumann [1930] and of Rothmann [1930], began to make it clear that the biological activity of many of the tissue extracts which had been studied by earlier workers was really due to their adenosine content.

Zipf [1930] showed clearly that the depressor substance which can be isolated from defibrinated blood, and which had been described by Freund [1920] as "Frühgift," was adenylic acid.

Bennet and Drury [1931] further investigated the production of heart block in the guinea-pig by injection of the adenyl compounds, and concluded that the amounts required to produce block were independent of the presence of other substances, and therefore might be used as a method for the biological assay of the adenylic acid present in tissue extracts. Adenylic acid was thus recognized in nearly all tissues, especially in heart and voluntary muscle.

Wedd [1931] investigated the action of adenosine and allied compounds on the coronary flow of the perfused rabbit's heart. He noticed the difficulty of correlating this action with that upon the musculature of the heart, which he concluded must be of a different nature. The maximal dilatant effects could be obtained with amounts which caused little or no cardiac slowing. He also noticed that adenosine was a more powerful coronary dilator than adenylic acid obtained from muscle or yeast.

Hochrein and Keller [1931] compared various tissue extracts by their effects on the blood-pressure and the electrocardiogram, and concluded that the active principle of most of them was the same, but no details were given regarding the chemical nature of the active substances.

Subsequently, Drury [1932 *a*] revised his view that deamination was the essential factor in the activity of the adenyl compounds, and he attempted to explain the effects as partly due to the presence of phosphate radicles in the molecule. He found, for example, that orthophosphate increased the amplitude of the mechanogram, while pyrophosphate first decreased and then increased it. Both forms of inorganic phosphate, however, reduced the coronary flow. Drury therefore supposed that the beat might be influenced by (1) the adenyl complex acting on the coronary vessels, and (2) the phosphate radicles acting on the musculature. This, however, is not sufficient to account for the fact that adenylic acid and even adenosine may produce heart block indistinguishable from that caused by a.t.p. Moreover, in producing effects with inorganic phosphates, Drury had to employ doses far outside the range of those used in the case of the adenyl derivatives, so that the effects are scarcely comparable.

Ostern and Parnas [1932] estimated the adenosine content of various tissue extracts by perfusion of the frog's heart. They stated that a.t.p. was three times as active as adenosine, judging by the time taken for heart block to develop. Deuticke [1932] compared the activities of the adenyl compounds on the virgin guinea-pig's uterus. Following a short latent period, contraction was caused by adenosine, adenylic acid, and a.t.p. as well as by yeast adenylic acid and a related nucleotide

recently isolated by Embden from the heart. He emphasized that the maximum height of contraction was reached more rapidly with substances which contained more phosphorus in their molecule.

Marcou [1932] examined the cause of the lowering of blood-pressure by adenosine, and found that it was evidently largely due to dilation of intestinal and peripheral vessels. The volumes of the spleen and kidney were at the same time diminished.

Gaddum and Holtz [1933] tested adenosine, adenylic acid, and a.t.p. on the vessels of the lung. They found the cat more sensitive than the dog: in both, constriction occurred, with fall in lung volume.

There is, therefore, a considerable accumulation of knowledge about the biological actions of adenyl compounds, which ought to make possible a definite statement as to their rôle in the activity of any given tissue extract. Unfortunately, the substances tested by most of the authors quoted have been obtained from widely divergent sources, and it is difficult to correlate the changes which occur in the biological activity of the adenyl compounds with variations in their chemical constitution. For example, Drury suggested that ease of deamination is not the essential factor in their biological activity from a comparision of yeast adenylic acid and muscle adenosine. The former cannot be deaminated by enzymes, while the latter can, although only with difficulty, and under special conditions. In several of the papers the exact source of the compound tested has not been stated.

A series of experiments was therefore carried out using the various derivatives from the same mother substance, the naturally occurring adenosine triphosphate of skeletal muscle. This substance was prepared from rabbits' muscle as a calcium salt. The distribution of the phosphorus per mg. dried salt was as follows:

Free (inorganic) phosphorus	Nil
Labile P hydrolysable in 10 min.	0·096 mg.
Total P liberated by total hydrolysis	0·143 mg.

The ratio of labile to stable phosphorus is the theoretical one of 2 to 1 and the N : P ratio was found to be the theoretical one (5 : 3).

As the changes most easily produced in the natural nucleotide are deamination and the removal of the two labile phosphoric acid molecules, it is necessary to compare the biological activities of adenosine—and inosine—triphosphoric acids (a.t.p. and i.t.p.), and of adenylic and inosinic acids respectively. It is evident, however, from the earlier work, that so long as the pentose linkage with adenine is retained, certain of the

characteristic effects produced by the adenyl derivatives are still obtainable even when the ester linkage between the sugar and phosphoric acid has been broken. Although doubts have been expressed regarding the possibility of the removal of NH_2 from a.t.p., it is possible to obtain i.t.p. by nitrous acid deamination. The body so formed has a 4N : 3P ratio and retains the two labile phosphoric acid molecules. Adenylic and inosinic acids may be readily obtained by weak alkaline hydrolysis of a.t.p. and i.t.p. respectively. Adenosine may be obtained in the usual way by alkaline hydrolysis at 170°, while the purine-pentose linkage can be readily broken by acid hydrolysis.

For purposes of injection, salts were used which were obtained either as hydrolysed solutions of a.t.p., analysed for their content of the derivatives, or in the dried purified form, made up to the required watery dilution. In the former case, the procedure was briefly as follows.

A weighed amount of the pure a.t.p. was suspended in the hydrolysing solution, and heated in sealed glass tubes. When it was desired to obtain adenylic acid as the sole derivative of alkaline hydrolysis, the water was made sufficiently alkaline to neutralize the two labile phosphoric acid molecules which would be set free. The tubes were then heated at 100° C. for 2 hours. The hydrolysed solution showed on analysis that the rise in inorganic phosphate was fully accounted for by the liberation of the labile H_3PO_4, without encroachment on the stable H_3PO_4 of the adenylic acid molecule.

It was thus easy to obtain the adenylic acid content of the solution, which also contained the labile H_3PO_4 of the original a.t.p. in the form of inorganic phosphate. In experiments carried out for the purpose, it was found that this inorganic phosphate was quite inactive in the amounts in which it occurred in the hydrolysed solutions.

When it was desired to obtain adenosine by hydrolysis, a weighed amount of a.t.p. suspended in 16 p.c. ammonia was heated at 170° C. for 2 hours. Analysis showed that this procedure set free all three H_3PO_4 molecules and, since the glucoside linkage remains unbroken under these conditions, it could reasonably be assumed that the solution contained only adenosine plus inorganic phosphate.

The ammonia used in the solutions was removed by aeration, and the reaction was brought to *p*H 7·3 by a few drops of dilute hydrochloric acid.

Inosine triphosphate was prepared by deamination of a.t.p. with nitrous acid, and the doses referred to in the text were the weights of pure dry sodium salt used for injection. The salt may have had some very

slight admixture with non-deaminated a.t.p., but the ratio of N to P was approximately 4 : 3 instead of 5 : 3.

Inosinic acid was also used after separation as a pure barium salt, prepared in the laboratory after the method of Ostern. For some confirmatory tests, a sample of pure dry barium inosinate, kindly sent by Dr Levene, was also used. In all cases the barium was removed before the salt was used biologically, the sodium salt being that employed. Adenosine was also tested in the form of "Lacarnol," kindly supplied by Messrs Bayer. Contrary to the statement of Rothmann [1930] we found on repeated analysis that the lacarnol in our possession was phosphorus-free, and therefore could not be a nucleotide. Whatever other components it may contain, adenosine can be recovered from it in practically pure form.

The actions of the salts on different test objects will now be described in order.

Blood-pressure.

(1) *Adenosine triphosphate and inosine triphosphate.*

The rabbit was largely used, but occasionally also the cat, the actions in the two animals being very similar. The blood-pressure was measured by a cannula tied in the carotid artery, and injection was made slowly

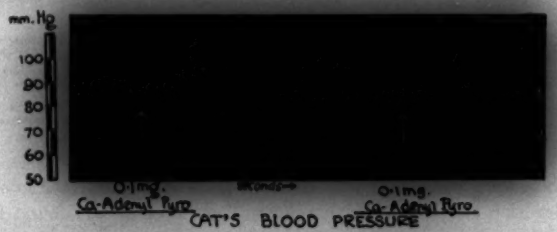

Fig. 1. Effect of injection of 0·1 mg. adenosine triphosphate on cat's blood-pressure. On repetition of the injection, the blood-pressure rise alone is seen.

into a cannula in the jugular vein. The time taken to inject was gauged so as not to affect the blood-pressure, and was about 7 sec.

The usual fall in blood-pressure follows injection of both these substances, but so long as the labile phosphoryl linkage is retained in the molecule, there is frequently to be seen a transient rise in pressure preceding the fall. These effects persist after atropinization.

A typical example may be given (Fig. 1). On injection of 0·1 mg. of adenosine triphosphate into the jugular vein of a cat after a latency of 7 sec., there was a rise in pressure of 20 mm. of mercury, followed at once by a fall of 18 mm. below the normal level. When a second injection is given rapidly after the first one, the rise in blood-pressure alone may be obtained.

The deaminated derivative is much less active, and doses five to ten times as great as those used with the mother substance are necessary to produce the effects. But the transient rise and subsequent fall are both evident when sufficient amounts (say 1 mg. of the dried salt) are injected.

(2) *Adenylic acid and inosinic acid.*

The well-known fall in blood-pressure was seen following injection of adenylic acid, but rarely with any evidence of the initial rise, which generally appears to indicate the presence of the labile phosphate in the molecule. After deamination the same decrease in activity is to be observed as in the case of the mother substance. Inosinic acid resembles adenylic acid in producing only the fall in pressure. The most inactive specimen of inosinate was that prepared by Dr Levene, which only produced a very slight fall in doses of 2 mg. Bennet and Drury [1931] described inosinic acid as quite inactive in their experience.

(3) *Adenosine.*

The typical fall in blood-pressure was obtained after injection of 0·1 mg. of pure adenosine and, judging by the duration of the fall, it appeared to be the most active of all the non-deaminated derivatives.

(4) *Adenine and hypoxanthine.*

These were tested after neutralization of the acid hydrolysed solutions of adenosine and inosine triphosphate. They were always found to be inactive.

HEART.

Rabbits' hearts were perfused using a modification of Gunn's apparatus, the coronary flow being estimated by allowing the fluid to fall into a bucket which tipped when it contained 1·5 c.c. In order to construct suitable graphs to show the relative activities of the various bodies as coronary dilators, the reciprocal of the time taken for 1·5 c.c. to perfuse was used as a measure of the rate of flow. The rate measured before injection of the drug was taken as 100, and the increase in this rate during

every second following injection was calculated as a percentage. The figures so obtained were plotted in the manner seen (Fig. 2), and all the graphs shown were constructed from the average figures of numerous experiments.

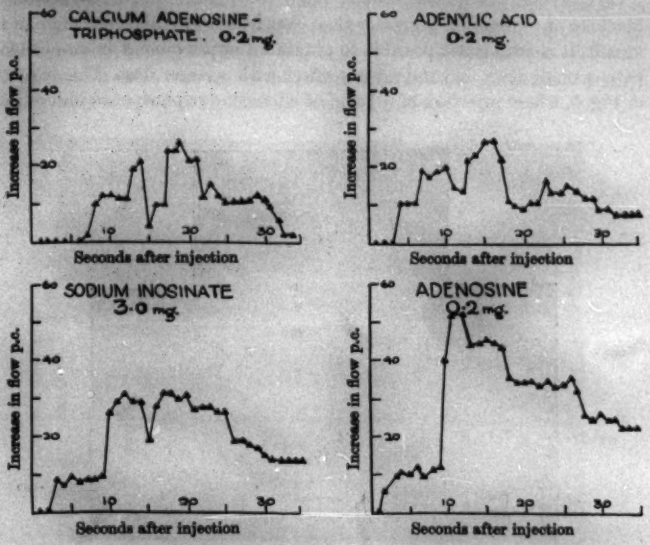

Fig. 2. Graphs showing the percentage increase in coronary outflow in perfused rabbits' hearts after injection of adenyl derivatives.

(1) *Adenosine triphosphate and inosine triphosphate.*

When 0·2 mg. of a.t.p. was injected into the side-piece of the cannula there was a latent period of 7 sec., after which an increase in the flow commenced which reached its maximum in 19 sec. The maximum increase was 26 p.c. above the normal, and in 33 sec. the flow had almost returned to normal. Very similar figures were obtained, using i.t.p., except that about ten times the dose was necessary to produce the effect. The graph is not shown because only five experiments were carried out with this drug.

Some workers have described improvement of the action of the heart by the adenyl compounds, while others only mention inhibition. This

appears to be because the action on the musculature and conducting system is really always depressant, while the improvement in the beat is secondary to the increased coronary flow. The reasons on which this statement is based are the following.

(1) In the mammalian heart, since the dose required to produce blocking of conduction is greater than that which will dilate the coronary vessels, it is sometimes possible to obtain an improvement in amplitude with a small dose, and the reverse effect with a larger dose. This is seen in Fig. 3, where injection of 0·1 mg. of adenosine triphosphate induced a

Fig. 3. 0·1 mg. adenosine triphosphate produced "heart block," while in the same heart 0·01 mg. dilated the coronary vessels and improved the beat.

"heart block," while in the same heart a dose of 0·01 mg. dilated the coronary vessels and improved the beat. Unfortunately a record of the coronary output was not kept in this case. Some of the improvement in amplitude in this record is doubtless due to the increased rate of beating, as pointed out by Dale.

(2) An increase in amplitude of the beat was only seen after the coronary outflow had been increased. The improvement followed a short latent period during which there was no coronary dilatation, or the beneficial effects of the dilatation had not been felt. During this period the "blocking" effect might appear (Fig. 4).

(3) In a heart where the coronary arteries were already fully dilated

no increase in amplitude of the beat has been seen to follow injection of the adenyl compounds.

(4) In the course of a large number of experiments in which the frog's heart was perfused with the Straub-Führer cannula the adenyl compounds were always seen to produce interference with conduction of the beat, and diminution in amplitude, and never an improved action of the heart.

It may here be mentioned that a.t.p. was the most active of the adenyl group in producing heart block in the frog's heart.

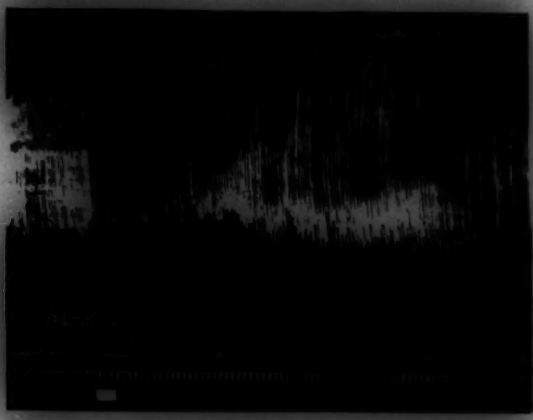

Fig. 4. The typical effect of adenosine triphosphate on the perfused mammalian heart.

The typical action of a.t.p. on the mammalian heart is seen in Fig. 4. This shows the combination of the blocking which is a direct action on the conducting system, and the improvement in the beat following the increased coronary flow. In this case there is very little alteration in the rate of beating, and any increase which there may be is not sufficient to account for the improved amplitude.

A similar picture, using ten times the dose of a.t.p., is given by i.t.p.

(2) *Adenylic acid and inosinic acid.*

The dilatation of the coronary vessels produced by adenylic acid resembles that caused by a.t.p. (Fig. 2), except that the increase in flow began 3 sec. earlier, and after 35 sec. was still 8 p.c. above the original

value. This may be taken as evidence of somewhat greater dilatant activity.

The graph obtained with sodium inosinate is very similar, but the effective dose was 3 mg., while 0·2 mg. was the dose employed with the non-deaminated compounds.

Adenylic acid and inosinic acid both produce heart block in the perfused amphibian and mammalian heart, but the loss of the labile phosphate from the molecule causes a loss of activity in this respect, as compared with a.t.p. and i.t.p. The criteria of this were the duration of the block in the rabbit's heart, and the time taken for the block to develop in the frog's heart.

(3) *Adenosine.*

Removal of both labile and stable phosphate groups from the molecule of adenosine triphosphate still further increases the dilatant activity upon the coronary arteries. Thus the most active of our series was found to be the adenosine prepared by alkaline hydrolysis (Fig. 2). The increased flow begins after 1 sec., reaches 52 p.c. above the normal in 12 sec., and after 35 sec. is still 22 p.c. above normal.

Using the same criteria as in the case of adenylic acid, adenosine appears to be the least active of the series in producing heart block. If its action in the intact animal be similar, this may help to explain the clinical popularity of adenosine, since it has the greatest margin of safety between the dose which dilates the coronary vessels and that which produces heart block. Nevertheless, Honey, Ritchie and Thompson [1930] have described dangerous interference with conduction in the human heart following intravenous injection of 50 mg. of adenosine.

(4) *Adenine and hypoxanthine.*

These were quite inactive on amphibian and mammalian hearts.

ISOLATED SMALL INTESTINE.

(1) *Adenosine triphosphate and inosine triphosphate.*

A.t.p. has a unique action on the small intestine which does not appear to have been mentioned in the literature. Like the other adenyl compounds it produces a fall in tone in concentrations as low as 1 in 500,000. But in many cases, and specially if the tone be initially low, it produces and maintains a subsequent rise in tone (Fig. 5). This increase in tone is sometimes very striking, and has been seen in a gut which showed no sign of life until the a.t.p. was added to the solution.

During the action of the drug the rate of contraction is not affected and, with the general increase in tone, the amplitude of the single contractions is decreased. Usually slightly higher concentrations (say 1 in 50,000) are required to bring out the tonic effect.

I.t.p. inhibited the gut in concentrations ten times as great as those in which the mother substance was active, but was never seen to produce increase of tone.

Fig. 5. Increased tone caused by adenosine triphosphate acting on small intestine in concentration of 1 in 50,000.

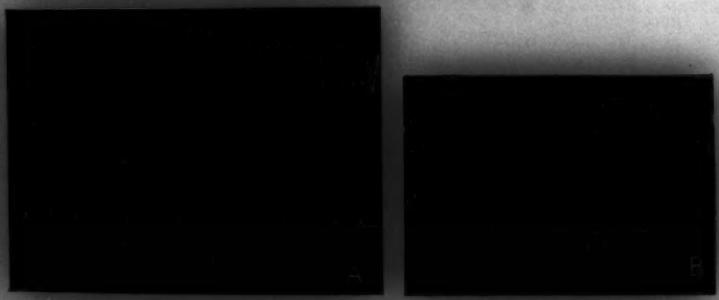

Fig. 6. A. Shows the inhibition followed by increase in tone of the intestine caused by a.t.p. in a concentration of about 1 in 10,000. Time = 5 sec. B. Shows how after hydrolysis in neutral solution (adenylic acid being formed) only the inhibition appears.

(2) *Adenylic acid and inosinic acid.*

Removal of the labile phosphate from the molecule of a.t.p. leaves the power of inhibiting the gut slightly weaker, while the subsequent increase in tone is no longer to be observed except very rarely with larger

doses. Comparison of Fig. 6 A and B shows how hydrolysis brings about this alteration in activity.

Inosinic acid gave results similar to adenylic acid in doses five times as great, only the inhibition of the gut being seen.

(3) *Adenosine.*

Adenosine inhibits the gut in concentrations of 1 in 50,000. It is the least active of the non-deaminated adenyl derivatives in this respect. It has only once been seen to produce increase in the tone of the gut, and that was with an unusually excitable specimen.

(4) *Adenine and hypoxanthine.*

These substances showed no action on the gut.

UTERUS.

The isolated virgin guinea-pig's uterus was used after the technique of Sawasaki [1925], as recommended by Deuticke [1932].

(1) *Adenosine triphosphate and inosine triphosphate.*

With a tension of 6 g. on the cornu, a.t.p. produces tonic contraction in concentrations of 1 in 500,000, or even less. The action does not appear to be as completely reversible as Deuticke suggests. Often the cornu starts to contract at a high level of tone after the specific action of the substance has passed off, and it is difficult to abolish these rhythmic contractions even by washing out the fluid. Since the uterus must be quiescent before a test can be made, the contractions may make it impossible to use the same preparation for testing a second specimen.

I.t.p. must be present in ten times the concentration of a.t.p. before it can produce increase in tone.

(2) *Adenylic acid, inosinic acid, and adenosine.*

Removal of phosphate from the molecule decreases the activity of these compounds on the uterus, so that a.t.p. is the most active of the non-deaminated series, and adenosine the least active.

Inosinic acid appears to have about one-tenth the activity of adenylic acid on this test object.

(3) *Adenine and hypoxanthine.*

These showed no activity on the uterus.

Some experiments were made in order to discover whether the adenyl compounds are freely dialysable in active form from the tissues where they naturally occur. Analyses of the fluids inside and outside a collodion tube containing a saline muscle extract, which had been allowed to dialyse against a Ringer-Locke solution for varying periods, showed that equilibrium was reached in the case of the adenyl compounds in about 2 hours, and that this time was unaffected by the presence of the colloid material in the muscle extract. Of course, if the glycolytic ferment were not inactivated by boiling, the ordinary enzymic changes would go on in the extract, and the a.t.p. would lose at the least some of its labile phosphate before it could escape into the outer fluid.

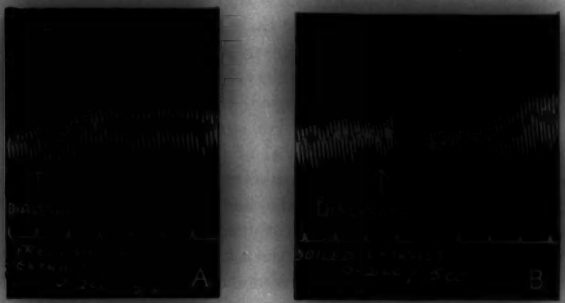

Fig. 7. A. Shows the effect of adding 0·2 c.c. of a 2 hours' dialysate of fresh saline extract of rabbit's muscle to 15 c.c. Ringer-Locke solution in which intestine was contracting. Time = 5 sec. B. Shows the effect of adding 0·2 c.c. of a dialysate of a similar extract in which ferment changes had been inhibited by boiling.

Note the much stronger activity seen in B.

The figure shows that, in the extract in which ferment action had been checked by boiling, the adenyl compounds can escape in biologically active form (Fig. 7). It is therefore possible that they may do so in the living animal, and, as we have pointed out, Zipf [1930] has shown clearly that the action of defibrinated blood plasma in lowering blood-pressure is due to its content of adenylic acid, which must consequently be always present there in small quantities during life.

The question thus arises as to whether the biological activities which the adenyl bodies show under experimental conditions have any counterpart in their normal activity in the living animal.

At the present time we can do no more than guess at the answer to this question. If we consider broadly the actions of all these compounds, two

facts are made fairly clear. In the first place complete loss of biological activity does not occur until the pentoside linkage in the molecule has been broken down. This has been pointed out recently by Ostern and Mann [1933], who note that the presence of phosphate in the molecule is not essential for the possession of biological activity. These authors say, however, in common with most workers, that deamination of the substances inactivates them completely. This we do not believe to be always the case. In fact, as far as we can see, the adenyl compounds never become completely inactive in the tissues, since deamination and removal of the phosphate is the greatest amount of destruction which they are likely to undergo.

But it is true in the second place that these compounds do suffer changes which very markedly alter their biological activity, and these changes occur constantly during muscular activity. The addition of phosphate to the molecule increases the activity of the compounds where tonic and muscular effects are concerned. Examples of this are the effects seen on the heart muscle, the intestine, and the uterus.

On the other hand, the removal of phosphate from the molecule seems to increase the activity of the substances as regards ability to dilate blood vessels. This has been seen in the case of the general blood-pressure, and most strikingly in that of the coronary vessels.

Possibly the tonic properties of the a.t.p. are made available for the muscle during anabolism and rest, while the early removal of the labile phosphate from its molecule during activity brings into play the more powerful dilatant actions of adenylic acid and adenosine. We should thus have a local adjuvant to the vaso-dilatation of activity in the normal breakdown products of metabolism.

It would seem, by reason of the extreme rapidity of all the biological actions, that the effects are really produced by alterations in the colloids at the surfaces of the cells concerned. A hundredth part of a milligramme of a.t.p. which succeeds in dilating one of the coronary arteries as it is whirled past at high pressure in a great volume of fluid has certainly not had time to take part in an elaborate metabolic process. It is this extraordinary rapidity of response, as well as of chemical change, which makes the approach to the problem so very difficult.

Summary.

1. Adenosine triphosphate, the naturally occurring adenyl compound, is more active than any of its derivatives in producing interference in conduction in the heart, and increased tone in the isolated virgin guinea-

pig's uterus. The removal of phosphate from its molecule does not lessen its activity so markedly as deamination.

2. A.t.p. has a tendency, which rarely appears in any of its derivatives, to increase the tone of the small intestine, after the preliminary depression which is also produced by the other active adenyl compounds. Deamination, and, to a less extent, the successive breaking off of the labile and stable phosphate linkages, lessen its depressant activity on the intestine.

3. Removal of phosphate from the molecule of a.t.p. increases its ability to lower blood-pressure and to dilate the coronary vessels. Thus the order of activity of the adenyl series is adenosine, adenylic acid, and a.t.p. Deamination greatly lessens, but does not destroy, this vaso-dilatant power.

4. A combination of the heart block and coronary dilation may give an explanation of some of the inconsistent behaviour of the so-called "heart hormones."

5. Biological activity finally disappears from the compounds when the pentose is split off from the purine base.

I gratefully acknowledge the advice and assistance of Prof. T. H. Milroy, given throughout the whole of this work.

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THE EFFECT OF INSULIN AND OTHER FACTORS ON IODO-ACETATE HYPERGLYCÆMIA.

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In a preliminary communication [1932] I showed that iodo-acetic acid, in toxic doses, caused a fairly pronounced hyperglycæmia in rabbits. This observation has also been made by Neuss [1931] and Hikiji [1932]. In addition, I observed that iodo-acetate was antagonistic in its action to insulin. The following experiments have been carried out in an attempt to elucidate the basis of this effect.

Experiments on the intact animal.

In all cases the acid used was recrystallized from hot petroleum ether and melted sharply at 82° C. (uncorr.). The necessary amount was dissolved in distilled water and neutralized to phenol red. Control experiments showed that it did not interfere with the method of sugar estimation used (Hagedorn and Jensen), even when added to blood in relatively large amounts. The animals used were rabbits which had been starved overnight, and the injection was subcutaneous.

Doses of various sizes were employed and were found to produce different results. The minimal lethal dose was related to the weight of the animal and was 60–70 mg. per kg. body weight. With smaller doses than these, no change was noticed.

With amounts of over 100 mg. per kg., two separate effects were observed. One was on the nervous system and consisted in a general depression of the higher centres. The rabbits first showed fatigue and their chins rested on the floor; this was succeeded by a stage of drowsiness and weakness, in which they lay on their sides; finally, after gasping respirations, they died. The other effect was the well-known one of marked muscular stiffness and rigor, often supervening before death. At death

the animal was usually in complete rigor. With lethal doses of less than 100 mg. per kg. the muscular effect was not often seen, the other alone being marked. The simple glycolytic mechanism of the brain [Ashford and Holmes, 1929] appears to be easily affected by small doses of the acid. In a few cases the heart was suddenly paralysed, but this often occurred after the other symptoms had been present for some time.

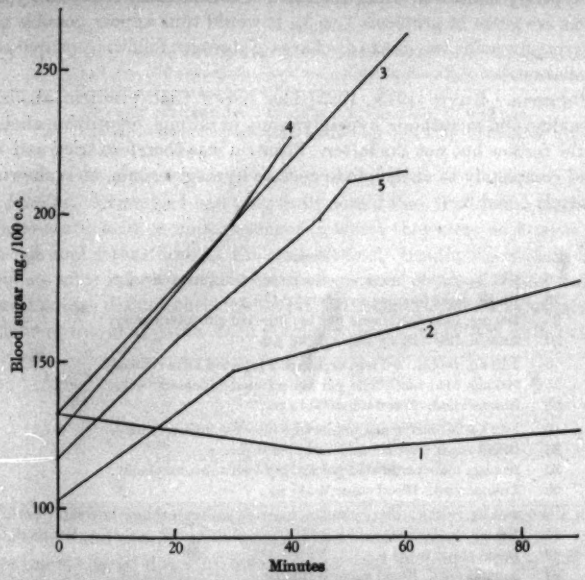

Fig. 1. Influence of iodo-acetic acid on the blood-sugar level. The dosage, in terms of mg. per kg., was respectively: No. 1, 50; 2, 70; 3, 80; 4, 120; 5, 200. The acid was injected at the beginning of the experiment, only No. 1 surviving.

In all cases except two, if the blood sugar rose, the animal died. The same degree of hyperglycæmia occurred with all doses above 80 mg. per kg., but with smaller doses it was not usually quite so marked (Fig. 1). It thus seemed that some process was set in motion by lethal doses of iodo-acetate which led, among other things, to hyperglycæmia. The possibility at once presented itself that this was due to interference with the centre in the pons which controls carbohydrate metabolism

[Donhoffer and Macleod, 1932] and was caused, in part at least, by mobilization of liver glycogen, probably by sympathetic impulses. The effects of ergotoxine were therefore tried.

Ergotoxine[1]. This was injected intravenously in 5–6 mg. doses as ergotoxine ethanesulphonate, dissolved in dilute acetic acid. The results obtained were not as clear-cut as could have been wished, but showed that the hyperglycæmia was either abolished or considerably reduced. Typical figures are given in protocols 1 to 3. It would thus appear possible that the hyperglycæmia was due to discharge of glycogen following sympathetic stimulation.

Pituitrin. Burn [1915, 1923] has shown that pituitrin abolishes adrenaline and anæsthesia hyperglycæmia in rabbits, ergotoxine abolishing the former but not the latter. Pituitrin was therefore tried and was found completely to abolish iodo-acetate hyperglycæmia, as is shown in protocols 4 and 5.

Exp. No.	Min.	Remarks
1	0	1·47 kg. rabbit. 6 mg. ergotoxine injected intravenously.
	25	Blood sugar (cardiac puncture) 0·155 p.c.
	35	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	50	Animal died. Blood sugar 0·139 p.c.
2	0	2·10 kg. rabbit. 5·5 mg. ergotoxine injected intravenously.
	25	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	85	Animal died. Blood sugar 0·113 p.c.
3	0	1·42 kg. rabbit. 6 mg. ergotoxine injected intravenously.
	25	Blood sugar (cardiac puncture) 0·164 p.c.
	30	200 mg. iodo-acetic acid per kg. injected subcutaneously.
	60	Animal died. Blood sugar 0·211 p.c.
4	0	2·14 kg. rabbit. 4 c.c. pituitrin injected subcutaneously into right flank.
	20	200 mg. iodo-acetic acid per kg. injected subcutaneously into left flank.
	23	Blood sugar 0·138 p.c.
	83	Animal died. Blood sugar 0·102 p.c.
5	0	2·01 kg. rabbit. 4 c.c. pituitrin injected subcutaneously into right flank.
	15	200 mg. iodo-acetic acid per kg. injected subcutaneously into left flank.
	19	Blood sugar 0·136 p.c.
	56	Animal died. Blood sugar 0·122 p.c.

Burn [1915] concluded that in rabbits pituitrin acted directly on the liver cells, since it abolished anæsthesia hyperglycæmia which ergotoxine did not do. Since, however, Evans, Tsai and Young [1931] have shown that anæsthesia hyperglycæmia is almost entirely due to the

[1] I am indebted to Prof. J. H. Burn for much useful information with regard to ergotoxine.

liberation of adrenaline, it is fair to assume that pituitrin acts like ergotoxine in abolishing the action of the sympathetic on the liver. Iodo-acetate has no effect on the breakdown of glycogen in slices of isolated liver as the figures in protocol 6 show. Thus it would seem that the hyperglycæmia has its origin largely in liberation of liver glycogen, which is almost certainly mobilized by the sympathetic.

Exp. No.	Remarks
6	Effect of iodo-acetate on liver glycogenase. 2 g. of liver were suspended in 1 c.c. of water, estimations being done on the whole suspension. Initial glycogen content 1·67 p.c. Glycogen content after 2 hours' incubation at 38° C. 0·48 p.c. Glycogen content after similar incubation with 1 mg. of (neutral) iodo-acetic acid 0·48 p.c.

Insulin. Although I had previously found that this was antagonistic to iodo-acetic acid, in no experiment in which they were both given was the minimal lethal dose of the acid altered. Insulin injected in large amounts after iodo-acetic acid had been allowed to act in the animal for a few minutes did not check the hyperglycæmia. The smallest lethal dose of iodo-acetic acid given during deep hypoglycæmia was sufficient to

Exp. No.	Min.	Remarks
7	0	1·91 kg. rabbit. 2 units insulin injected.
	125	Convulsions. Blood sugar 0·048 p.c.
	130	70 mg. iodo-acetic acid per kg. injected.
	160	Died. Blood sugar 0·110 p.c.

raise the blood sugar (protocol 7). A mixed effect was obtained if iodo-acetic acid and insulin were injected simultaneously. In most of these experiments large doses of insulin were used (6 units). With doses of iodo-acetate above 100 mg. per kg. hyperglycæmia developed before death and the insulin was without effect. As the dosage of iodo-acetate was reduced the action of insulin came to the fore, and, although the animal succumbed, no hyperglycæmia, or else hypoglycæmia, was found. It was quite plain which drug was exerting an effect, as, when iodo-acetate was predominant, no insulin convulsions were seen but solely the weakness effect mentioned above, and glucose injections did not save the animals. On the other hand, if they went into typical insulin convulsions, they would usually recover with glucose. The results are shown graphically in Fig. 2. The interesting point about them is that there appears to be here a peripheral hypoglycæmia insulin effect which is not affected by

iodo-acetate in toxic doses. The same thing was found in the perfusion experiments mentioned later.

It had been considered at one stage that iodo-acetate and insulin might react together and mutually inactivate each other, though this was improbable on chemical grounds. This was disproved by mixing small amounts of insulin with large amounts of iodo-acetate and allowing them

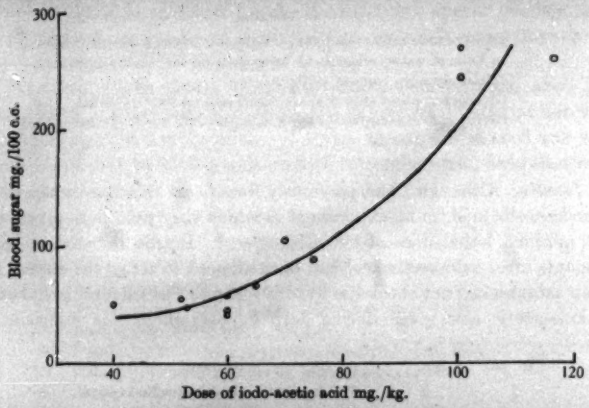

Fig. 2. The influence of the simultaneous injection of insulin (6 units) and iodo-acetic acid (varying amounts) on the blood-sugar level. The blood-sugar values given are those at death or the lowest amounts found.

to stand together for varying lengths of time. On decomposing the iodo-acetate with thiosulphate, the insulin was found to be unimpaired in activity.

Perfusion experiments.

It seemed possible that, in addition to causing hyperglycæmia by mobilizing liver glycogen, iodo-acetate might inhibit the utilization of glucose in the tissues. In order to investigate this, a series of perfusion experiments was undertaken, using rabbits' hindlimbs.

250 c.c. of mixed arterial and venous blood were collected from five to seven rabbits and defibrinated. Usually a small amount of glucose was added. The perfusion was done by gravity at a pressure of 100 mm. Hg. From a reservoir the blood entered a glass spiral, where it was heated, and then traversed the preparation; the outflow passed through a flow

counter and a von Euler and Heymans' oxygenator [1932], being finally returned to the reservoir by a pump worked by hand.

Rabbits of about the same weight were used for the preparation. The one employed was anæsthetized with urethane (1·25 g. per kg. intraperitoneally), the abdominal aorta was cleaned and the lumbar, inferior mysenteric and other arteries in the neighbourhood were ligatured. The aorta was opened and a cannula inserted just below the renal arteries. The animal was then cut transversely at this level and the cannula connected to the apparatus. The preparation was kept warm by a heated plate below and hot-water bottles above and was completely wrapped up in a duster. A thermometer was placed between the preparation and the hot plate and the temperature there was kept at 40° C. As the pump and the glass spiral originally contained approximately 50 c.c. of saline, the total volume of circulating fluid was 300 c.c. The blood was allowed to circulate completely once, and then the first sample was taken and the blood sugar determined. The perfusion was allowed to continue for 1 hour and a further sample was taken. Usually 1–1½ litres of blood flowed through the preparation during that time. It had been hoped to compare the sugar utilization in two separate 1 hour periods in the same preparation, one before, and one after adding iodo-acetate, but it was found that though the sugar utilization was fairly constant from preparation to preparation during the first hour, it always fell away after that time. It was therefore decided to determine the sugar utilization for 1 hour and compare those of different preparations. No difficulty was experienced with pressor substances. During the first few minutes the rate of flow fell owing to vaso-constriction in the preparation, but this never lasted longer than 5 min., and after that a uniform rate was obtained. It was often noticed that iodo-acetate increased the permeability of the capillary walls so that the total volume of blood in the circuit was markedly reduced by the end of 1 hour.

Owing to the expense, not many experiments were performed, but sufficient data have been collected to give a definite answer.

Experiments were first undertaken to compare the sugar utilization of the preparation with and without added iodo-acetate. When the acid was present, it was in a final concentration (allowing for the water content of the preparation) of from 1 : 2000 to 1 : 6000. The results are shown in Table I. It will be seen that in five of the six experiments in which iodo-acetate was present, the utilization of sugar was considerably depressed. Blood glycolysis did not account for more than one-quarter of the sugar disappearing in the control experiments.

TABLE I. Sugar utilization of perfused hindlimbs in the absence and presence of iodo-acetate.

Conc. of iodo-acetic acid (neutral)	Weight of preparation g.	Mg. sugar used per kg. limbs per hour
—	790	111
—	665	110
—	954	68
	Average	96
1 : 6000	777	37
1 : 2100	816	7
1 : 2700	630	0
1 : 2200	700	27
1 : 3500	694	26
(1 : 2200	847	85)*
	Average	19

* This value has not been included in the average.

Further experiments were undertaken in which insulin was present. In each case 5 units were added to the blood before the experiments started. The results are shown in Table II. As has been found by many

TABLE II. Sugar utilization of perfused hindlimbs in the presence of insulin (5 units), and the absence and presence of iodo-acetate.

Conc. of iodo-acetic acid (neutral)	Weight of preparation g.	Mg. sugar used per kg. limbs per hour
—	850	177
—	760	107
—	754	204
—	770	168
	Average	164
1 : 3600	742	113
1 : 2700	700	131
1 : 2800	718	13
1 : 2800	739	49
1 : 2900	798	113
	Average	84

other workers, the presence of insulin in the control experiments almost doubled the sugar utilization. The results obtained with iodo-acetate in this series were not so clear-cut, but served to show that the utilization of sugar, though lessened, was not nearly so much inhibited as when insulin was absent. This would seem to agree with the results previously referred to, in which lethal doses of iodo-acetate in the intact animal did not prevent insulin hypoglycæmia.

Discussion.

A perusal of the literature shows that there is little agreement as to the fate of the blood sugar in the peripheral tissues, especially where insulin is concerned. Best, Hoet and Marks [1926] found that in the absence of insulin no glycogen was formed in muscles even if much glucose was present in the blood stream, and this has been confirmed by Choi [1928] and by Markowitz, Mann and Bollman [1929]. On the other hand, Long and Horsfall [1932] found an increase of muscle glycogen under these conditions (though more was laid down if insulin was present), and Cleghorn and Peterson [1932] found the muscle glycogen of decerebrate eviscerate cats to recover after tetanic contraction. Thus, though it would appear that under the action of insulin the increased glucose disappearance is largely seen as glycogen formation, with no insulin present the relative amounts of glucose undergoing oxidation and storage are not definitely known.

From the figures of Best, Dale, Hoet and Marks [1926], it is seen that under the action of insulin, roughly half the sugar used by the muscles was burnt and half stored. The same was found by Cori and Cori [1928]. Although it is not safe to argue from so few figures as those above, it may be not without significance that the degree of inhibition of sugar utilization in Tables I and II is approximately the same, which might be interpreted as meaning that in the absence of insulin oxidation of sugar alone occurs, and that both in the presence and absence of insulin this is the sole mechanism affected by iodo-acetate.

It is generally considered that iodo-acetate inhibits the formation of lactic acid from glucose and glycogen. Recently, however, Brücke [1933] has reported that it will also prevent the formation of glycogen from glucose by yeast. Goldblatt [1933] has also shown that non-lethal doses prevent the accumulation of liver glycogen which normally follows the administration of adrenaline and insulin in young rabbits, and Hikiji [1932] has found that daily injections of small amounts of bromo-acetic acid caused, among other things, a disappearance of muscle glycogen and death in a few days. It is possible that in my experiments iodo-acetate inhibited glycogen formation as well as glucose oxidation. But it does not appear to be permissible to argue from this that lactic acid is a normal intermediate between blood sugar and muscle glycogen, as it has often been shown that lactic acid in the blood stream cannot be converted into muscle glycogen [*e.g.* Long and Horsfall, 1932; Eggleton and

Evans, 1930]. The only known possible intermediate is dihydroxyacetone [Kermack, Lambie and Slater, 1929].

It is hoped in future experiments to determine by direct analysis, using a different type of preparation, to what extent muscle glycogen formation is inhibited by iodo-acetate, and if possible what intermediate compounds are involved.

Summary.

1. Lethal doses of iodo-acetate always cause a hyperglycæmia, which is often marked.

2. This is largely inhibited by ergotoxine and completely annulled by pituitrin, without altering the toxicity of the acid.

3. Iodo-acetic acid usually reverses or prevents insulin hypoglycæmia. Using appropriate doses, however, it is possible to obtain a hypoglycæmia without affecting the toxic effect of the acid.

4. Iodo-acetic acid markedly inhibits the utilization of sugar by the tissues in the absence of insulin. When insulin is present, this inhibition is not so pronounced.

5. The conclusion is drawn that iodo-acetic acid causes hyperglycæmia by mobilizing liver glycogen and interfering with the disappearance of sugar in the tissues.

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THE CARBOHYDRATE METABOLISM OF THE KIDNEY.

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(*Received August* 18, 1933.)

The investigation described in this paper was undertaken to determine the rate at which the kidney used carbohydrate, the relationship of carbohydrate metabolism to urine formation and to total renal metabolism as assessed by the oxygen consumption.

An obvious way of measuring the rate of carbohydrate usage would be to estimate the sugar in blood from the renal artery and vein and multiply the difference by the rate of blood flow. Consideration of the possible rate of carbohydrate metabolism, based on measurement of renal oxygen consumption [Barcroft and co-workers, 1905, 1910; Bainbridge and Evans, 1914; Fee and Hemingway, 1928] and the blood flow, suggested that such a method could not be applied to the kidney because the difference in blood-sugar content would be too small for estimation. Neither would it take account of glycogen which might be stored in or liberated from the organ.

It was therefore decided to use the pump-lung-kidney preparation introduced by one of us [Hemingway, 1931] and to measure the total carbohydrate usage of the whole preparation. The usage due to the kidney can be found by making an allowance for the storage or liberation of glycogen and for the metabolism of the lungs.

Methods.

After preliminary experiments had confirmed the impossibility of estimating the difference in sugar content between the arterial and venous blood and the necessity for considering changes in the glycogen content of the perfused organs, the following methods and procedure of experiment were adopted.

(a) The pump-lung-kidney preparation and procedure of experiment.

The pump-lung-kidney preparation was set up according to the method previously described [Hemingway, 1931], but an improved pump was introduced into the circuit [Hemingway, 1933]. Other slight modifications of the original method were introduced during the course of the experiments. The apparatus was filled with alcohol when not in use and washed with water and saline immediately before use.

Two dogs were usually used for an experiment, one supplying blood and lungs, the other the kidney. The first dog, weighing 17–18 kg., was anæsthetized and about 450 c.c. of blood removed. This was whipped, twice filtered through organdie muslin [mesh = 0·01 in.] and a measured volume introduced into the apparatus.

Meanwhile a cannula was introduced into the pulmonary artery, the lungs were removed from the thorax and after weighing, perfusion was commenced. The lungs were accommodated on a large Büchner funnel and positively ventilated by a Schuster pump instead of being kept in a closed vessel, as originally described, because it was necessary during an experiment to remove samples of lung for glycogen estimation.

The second dog was anæsthetized while the lung preparation was being set up and the left kidney was removed and perfused as soon as the pulmonary flow was established. Simultaneously a slice of the right kidney was removed for glycogen estimation. When the blood flow through the kidney was established, and constant (usually within 2 or 3 min. of the attachment of the kidney to the apparatus) a portion of the lung was tied off and removed for estimation. Simultaneously, blood was taken for blood-sugar determination.

The output of the pump was constant in any one experiment and the fixed perfusion pressure to the kidney was regulated by passing some of the blood through a shunt directly to the lungs. This ensured that the flow through the lungs was constant and independent of that through the kidney.

The perfusion was allowed to continue for 40–50 min., and, at the end of the period, samples of blood, lung, and kidney were taken for estimation. The samples of lung and kidney were the counterparts of those taken for the first estimations. That the glycogen content of corresponding portions of the organs is similar is shown by Table I, and confirmation is given by Irving [1928 *b*] for the kidney.

TABLE I. Glycogen content of right and left kidneys and lungs.

Exp.		Glycogen mg./100 g.
18	Right lung	177
	Left lung	170
24	Right kidney	68
	Left kidney	70

The perfusion apparatus was then drained and the volume of remaining blood measured; the lungs and the kidney were weighed. The difference between the initial and final amounts of carbohydrate in the system when calculated gave the quantity of this material used by the kidney and lungs. Samples of urine were tested from time to time for reducing substance but none was found.

In other experiments the sugar used by the lungs alone was measured, and in these an exactly similar technique was followed except that the kidney was not added to the circuit.

(*b*) *Estimation of glycogen.*

As a result of experience a modification of the Pflüger method of glycogen estimation based on that described by Kerly [1930] was adopted. A fragment of tissue judged by eye to be about 4 g. was dropped into a previously weighed tube containing 5 c.c. of 60 p.c. KOH. The exact weight of the tissue was immediately determined and the tube placed in a boiling water bath. After about 2½ hours the alkaline digest of the tissue was cooled and neutralized by 60 p.c. trichloroacetic acid. The neutralized samples were made up to 25 c.c. and filtered. 20 c.c. of the filtrate were added to 80 c.c. of alcohol and allowed to stand in the ice chest overnight. The glycogen was separated by centrifuging and taken up in 15 c.c. of *N* H_2SO_4, and heated on a water bath for 2 hours. After hydrolysis the acid was neutralized by an accurately measured quantity of 60 p.c. KOH and made up to 25 c.c. Finally, samples were analysed for glucose by the Somogyi modification of the Shaffer-Hartmann method [Peters and van Slyke, 1932] and the glycogen values expressed in terms of reducing sugar in the hydrolysate.

(*c*) *Estimation of blood sugar.*

Blood-sugar determinations were made by the Somogyi modification of the Shaffer-Hartmann method on protein-free blood filtrate prepared after the method of van Slyke and Hawkins [1928]. Not only was it desirable to use the same method for the determination of reducing

substances in blood and in glycogen hydrolysates, but our experience indicated that this method was in many ways the most convenient in practice.

Results.

Separate consideration has been given to (*a*) the "true" metabolism of the lungs, making allowance for storage or release of glycogen, (*b*) the rate of disappearance of sugar from circulating blood and (*c*) the usage of sugar by the kidney.

(*a*) *The metabolism of the lungs.*

Previous investigations have been made on the metabolism of perfused lungs by Evans and Starling [1913], Patterson and Starling [1913] and Cruickshank and Startup [1930]. Patterson and Starling examined the sugar metabolism and concluded that the average rate of usage was 1·3–1·5 mg./hour/g. of heart muscle. These earlier investigations did not take into reckoning that some of the sugar disappearing from the blood might not be oxidized but, on the contrary, synthesized to glycogen. Cruickshank and Startup reopened the question with this point in view, and by making simultaneous measurements of oxygen consumption, respiratory quotient, and rate of sugar disappearance from the circulating blood, were able to measure with greater certainty than before the amount of sugar actually used and that stored or liberated. They gave 0·7–0·8 mg./hour/g. of heart muscle as the rate of usage. These results, as well as those of Patterson and Starling, are expressed in terms of heart muscle weight rather than in lung weight, because in their experiments the lungs tended to become œdematous, thus making the correct determination of lung weight difficult.

During the present experiments it was thought advisable, for two reasons, to redetermine the rate of lung metabolism, (*a*) glycogen changes were being investigated by a direct method instead of the indirect methods of previous investigators and (*b*) the method of pulmonary perfusion employed was dissimilar to those previously used.

The results of a typical experiment in which the rate of lung metabolism was measured are given in the accompanying protocol (see p. 373).

The diminution which occurred in the amount of circulating blood was attributed after further experiment to evaporation from the outer surface of the lungs. Such loss was minimized by covering the Büchner funnel with a damp cloth, but even so it was always sufficient to necessitate careful measurement of the residual blood at the end of an experiment.

Protocol of Exp. 24.

	Amount	Carbohydrate concentration	Total carbohydrate mg.
	Beginning of period.		
Lungs	94 g.	154 mg./100 g.	145
Blood	461·5 c.c.	112 mg./100 c.c.	516
			661
	After 46·5 min. perfusion.		
Lungs	100 g.	182 mg./100 g.	182
Blood	448 c.c.	94 mg./100 c.c.	421
			603

58 mg. of carbohydrate used by lung (average weight 97 g.) in 46·5 min. Rate of usage = 0·013 mg./g./min.

Three experiments were performed and the average of the three determinations gave the carbohydrate metabolism of the lungs as equivalent to 0·014 mg./g./min. The findings in the individual experiments are given in Table II.

TABLE II. Glucose usage of perfused isolated lungs.

Exp.	Wt. of lungs g.	Glucose usage mg./g./min.
3	100	0·014
23	126	0·015
24	97	0·013

This rate of metabolism is in good agreement with that found by Cruickshank and Startup, whose experiments, taking the lung weight as 87 p.c. of the heart weight [Evans and Starling, 1913], show a lung usage of 0·013–0·015 mg./g./min. The results are expressed in terms of lung weight and not heart weight, because the lungs were weighed at the beginning and the end of the experiment and little or no œdema was found. The changes in lung weight occurring in three successive experiments are given in Table III and are typical of the whole series.

TABLE III. Weight of lungs before and after perfusion.

Exp.	Wt. before perfusion g.	Wt. after perfusion g.
24	94	100
25	130	126
26	166	173

(*b*) *The disappearance of carbohydrate from circulating blood.*

Cruickshank and Startup [1932] investigated the rate at which sugar disappeared from blood which was mechanically circulated and oxygenated, and in a summary of their results stated that in 3 hours the

loss of sugar was 42·5 p.c. of the original total. On this account, it was considered whether a correction should not be applied to our results, making an allowance for sugar disappearing from the blood but not metabolized by the perfused tissues.

To obtain data for a correcting factor, similar experiments to those performed by Cruickshank and Startup were performed, but different samples of blood showed changes both greater and smaller in amount than their results indicated. Also, it was debatable whether it would be reasonable to make any comparison of changes occurring in blood circulating through tissues and in blood mechanically circulated and oxygenated, knowing that there are differences between such bloods, *e.g.* the difference in vaso-constrictor properties. Because of this, and our inability to obtain a constant figure, no correction has been made for carbohydrate disappearing from the blood but not metabolized by the tissues.

(*c*) *The glucose usage of the kidney.*

The results obtained for the kidney require little explanation. The total usage of the lungs and kidney was determined over a period and then an amount corresponding to the weight of the lungs was subtracted on the assumption that the rate of usage was 0·014 mg./g./min. Eight experiments were performed, using the described technique, and in Table IV are given the results of five, none of which showed any technical fault to careful scrutiny. In these experiments the rate of sugar usage varied between 0·014 and 0·099 mg./g./min. The figures are of the same order as those given by Irving [1928 *a*] for the glucose usage of minced rabbit kidney cortex which varied between 0·018 and 0·058 mg./g./min.

Table IV. Summary of experiments in which the glucose usage of kidney has been measured.

Exp.	Wt. of kidney g.	Glucose usage mg./g./min.	Urine c.c./min. (average)	Blood flow c.c./min. (max.)	Blood flow c.c./g./min.	Perfusion pressure mm. Hg
20	48·3	0·041	0·30	243	5·0	120
25	24·8	0·014	0·31	91	3·7	116
27	58·9	0·036	0·28	—	—	120
28	42·8	0·099	0·52	217	5·0	120
29	36·6	0·046	1·04	186	5·1	140

Discussion.

The results seem to invite the discussion of two main points, (*a*) the relationship of the rate of glucose usage to renal activity and (*b*) the ratio of carbohydrate metabolism to total metabolism in the kidney. It is not

easy to make a definite statement regarding the first of these points because the readings have been taken over a period during which renal activity might be varying. Nevertheless, in general the experiments in which a high glucose usage has been recorded were those in which there was a large blood and/or urine flow. Indeed if the weight of the kidney be taken into consideration the rate of glucose usage appeared to follow the rate of blood flow. This result is natural enough if the tubule cells are regarded, as they must be, as doing work in transferring materials and water from blood to tubule lumen and *vice versa*, but on the other hand, observations of the rapid flows in the kidney treated with cyanide or asphyxiated show that little reliance can be placed upon the rates of urine and blood flow as true indicators of renal activity.

The second point leads to a more definite conclusion. It will be realized that if the oxygen used by the kidney [Fee and Hemingway, 1928] is employed solely in the oxidation of carbohydrate, then the glucose usage should fall within the limits of 0·039 and 0·26 mg./g./min. The figures recorded here are much below these rates, but the ratios for maximal and minimal values, between the measured and the highest possible glucose usage, as calculated from the oxygen consumption, are in remarkable agreement as is shown in Table V.

TABLE V. Relationship between measured rates of glucose usage and those calculated from the oxygen consumption [Fee and Hemingway, 1928].

	Rate of glucose usage mg./g./min.		Ratio
	Measured	Calculated from oxygen usage	measured / calculated
Minimal	0·014	0·039	0·36
Maximal	0·099	0·260	0·38

This close agreement, which suggests that about one-third of the oxygen used is employed for carbohydrate metabolism, assuming that the carbohydrate which disappears is completely oxidized, may be purely fortuitous and the exact differentiation of the usage of oxygen between carbohydrate and other substances can only be found in an individual experiment by determining the R.Q. of the kidney. The difference between the two sets of figures measured and calculated is however so great (and is actually increased if the whole of the carbohydrate which disappears is not completely oxidized) that it is certain that the whole of the kidney metabolism is not due to oxidation of carbohydrate.

The early experiments of Bainbridge and Evans [1914] on the

R.Q. of the kidney in the heart-lung-kidney preparation show a quotient of less than unity and an average of 0·71, and suggest that the proportion of carbohydrate metabolism to the whole is very small.

Summary.

1. A method of measuring the carbohydrate metabolism of a perfused organ is described.

2. The carbohydrate usage of the perfused isolated lung, estimated as glucose, is 0·014 mg./g./min.

3. The carbohydrate usage of the perfused kidney varies between 0·014 and 0·099 mg./g./min.

4. About one-third of the oxygen used by the kidney is employed in the metabolism of carbohydrate.

The expenses of this research were defrayed in part by a grant to one of us (A. H.) from the Government Grants Committee of the Royal Society to whom our thanks are due.

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ON THE OCCURRENCE OF TWO KINDS OF HÆMOGLOBIN IN NORMAL HUMAN BLOOD.

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(*Received November* 6, 1933.)

In the fifth chapter of Barcroft's book on hæmoglobin he discusses its specificity, and suggests the possibility of more than one hæmoglobin in the same blood, the differences being located in the globin part of the molecule. The curious change in "span" (*i.e.* the distance between the bands for oxy- and CO-hæmoglobin) in the blood of the same rabbit makes Barcroft put the question, "Why and how should the globin of a rabbit's hæmoglobin alter on hæmorrhage?"

In limiting the problem to the unity and variability of hæmoglobin in one kind of blood, especially in human blood, we find that most methods of research on the specificity of hæmoglobin are not sensitive enough to detect the presence of, say, 10 p.c. of a modified hæmoglobin in 100 p.c. total. Only the estimation of the so-called alkali resistance, which gives enormous differences in blood of different species, might be sensitive enough. This paper describes the photo-electric measurement of the rate of denaturation of human hæmoglobin at *p*H about 12, as a means of differentiation between more than one kind of pigment in the same blood.

Method.

Von Krüger [1888, 1925, 1927], who first studied the rate of hæmoglobin denaturation in alkaline solutions ("Zersetzungszeit"), added 1/5 vol. *N*/4 NaOH to the solution of hæmoglobin and measured the time from this moment to the visual disappearance of the typical absorption bands. In this way he found the very large differences in "Zersetzungszeit" in the hæmoglobin of mammalian species, for instance for human blood 1 min., for rabbit's blood 30 min., for horse blood 80 min., for ox blood 24 hours. A more exact study was made by Haurowitz

[1929], who measured the rate of extinction in the region of the β bands (538–544) and also in the α region (572–580) by means of the spectrophotometer.

For production of the alkaline reaction he used the same process as von Krüger, viz. the addition of 1/5 vol. *N*/4 NaOH.

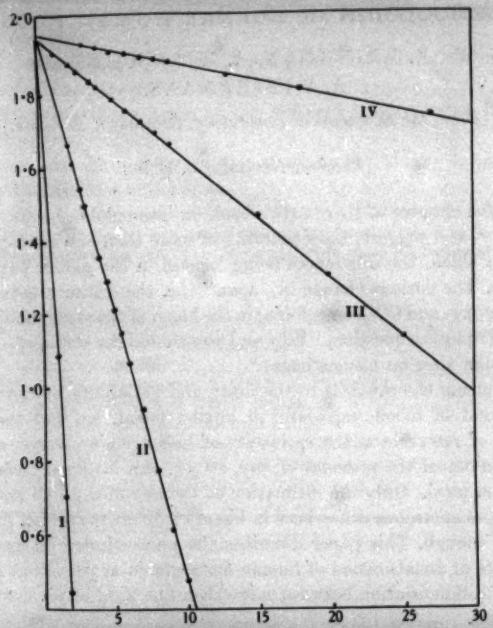

Fig. 1. Effect of *p*H on rate of alkali denaturation at 19·6° C. Vertical axis: log percentage unchanged hæmoglobin in total mixture; horizontal axis: time in minutes. (I) *p*H 12·12; (II) *p*H 11·89; (III) *p*H 11·66; (IV) 11·55.

The sensitivity of this method is very much dependent on the investigator's experience, and on the time which may be taken for each determination. In our opinion it will be difficult to estimate a decrease in extinction, smaller than that corresponding to a 2 p.c. change of oxyhæmoglobin to globin hæmochromogen. For our purpose we have tried to combine a good sensitivity with the exclusion of the subjective

factor, by using a photo-electric arrangement, and by keeping the conditions which govern the speed of reaction sufficiently constant.

The two factors on which the rate of alkali denaturation of a certain type of hæmoglobin is dependent are (*a*) the concentration of OH ions, and (*b*) the temperature.

Fig. 1 shows the influence of *p*H on the rate of denaturation at constant temperature. The hæmoglobin was obtained from a human blood, which did not contain more than 5 p.c. of the resistant form of hæmoglobin (to be described later). The ratio's log conc. HbO_2 : time are straight lines for about 80 p.c. completion of the reaction.

The solution of oxyhæmoglobin was made, by diluting oxalate blood with 0·15*N* Na_2HPO_4 solution, containing 1 p.c. of saponin, the final concentration of hæmoglobin being 1·36 p.c. This solution was centrifuged till it was quite clear and put into the absorption vessel of 1 cm. transverse diameter. The alkali denaturation was started by injection of 1 c.c. NaOH of various concentrations, by means of a precision syringe.

A special set of experiments showed that the described procedure enabled us to place the oxyhæmoglobin quickly in a medium of known and sufficiently constant *p*H; *p*H in each experiment was determined by the bubbling hydrogen electrode. Great care was taken to keep change of temperature in the reaction vessel within 0·1° C.

The temperature coefficient of protein denaturation being in many cases very large [Pauli, 1933], it is not surprising that a slight change in temperature has a definite effect on the rate of formation of globin hæmochromogen. In our experiments the temperature did not change over more than 0·1° C. in each measurement, although differences of temperature between separate experiments may be larger.

The colorimetric estimation of the rate of change of oxyhæmoglobin to alkaline globin hæmochromogen.

We used two methods, a bicolorimetric estimation without standard solutions and a photo-electric determination with calibration by means of known mixtures of HbO_2 and globin hæmochromogen.

The second method being the more exact one, it was solely used later on, although the bicolorimetric procedure too can give the rate of denaturation with sufficient sensitivity and may be briefly described for the use of those who wish to make analogous measurements.

Human, hæmolysed blood, containing 2·78 mg. Hb per c.c. was saturated with pure CO, made very clear by centrifugalization and divided in 3 × 5 c.c. in test-tubes *A*, *B* and *C*. Carboxyhæmoglobin is used, because its change on alkaline denaturation gives the most pronounced change in colour; the same observation has been made for the bicolorimetric determination of methæmoglobin [Clark, 1933]. To *A* is given 5 c.c. buffer solution so that the reaction is fixed at *p*H 12·00 at 18° C.; the tube is then placed at 37° C. for 15 min.,

the oxyhæmoglobin is thereby completely converted into the darker denaturation product. After addition of 5 c.c. distilled water to *B*, 5 c.c. of *A* and of *B* are filled in the lower and upper right cups of the colorimeter. *C* is placed in a 25° C. bath, together with a tube, containing 5 c.c. of the alkaline buffer solution. The contents of these two tubes are then quickly mixed and poured into the left colorimeter cup, fixed at 4 cm. distance and having a constant temperature circulating water jacket. From this moment the stopwatch is started, and colours are matched by making suitable optical mixtures of *A* and *B*, the total depths of liquid at both sides remaining 4 cm.

The best light for sufficient sensitivity was obtained by illuminating a piece of paper, brilliantly painted in the complementary colour of carboxyhæmoglobin, by a powerful Liesegang globoscope and sending the reflected light directly through the colorimeter cup; the light was turned off between measurements. The bicolorimeter readings are directly proportional to the amount of oxyhæmoglobin in total pigment; the method will indicate a change of about 3 p.c. of conversion. The results obtained were similar to those given by the photo-electric method which will now be described.

A method, suitable for our purpose, must be capable of distinguishing quantitatively between, say, a mixture of 95 p.c. globin hæmochromogen and 5 p.c. oxyhæmoglobin and a mixture of 94·5 and 5·5 p.c. respectively. We were not able to obtain a suitable monochromatic light of sufficient strength, but use a $CuSO_4$ filter, which cuts off the extreme red, which is not absorbed by globin hæmochromogen and to which the selenium cell is rather sensitive.

A 1000 c.p. Pointolite lamp is connected in series with an extra resistance and galvanometer; during the measurement an assistant regulates the current continuously at exactly 3 amp., which ensures sufficient steadiness of illumination and discards with the necessity of a differential method. An 8·0 lens concentrates the light on the surface of a selenium-oxyde "Sperrschicht" photo-electric cell, after it has passed through a $CuSO_4$ filter and the hæmoglobin solution (20 mm. of a 1·2 p.c. $CuSO_4$ solution). The selenium cell is connected in series with an E.M.F. of 300 mv. and a 10,000 ohm fixed resistance; the drop in potential over this resistance is measured by a Cambridge electrostatic valve potentiometer.

The sensitivity of this arrangement is seen in Fig. 2; for our purpose the most important change is that from 90 p.c. globin hæmochromogen and 10 p.c. oxyhæmoglobin to 100 p.c. of globin hæmochromogen. This gives for a 1·0 p.c. solution of human oxyhæmoglobin a drop of 28 mv., with an accuracy of at least 1 mv.; for the change from 80 to 100 p.c. globin hæmochromogen the drop is 61 mv.

If the concentration of hæmoglobin is doubled, the drop for the last 10 p.c. of the native pigment is only 20 mv., because the total absorption of light is too large. For this reason the best way is always to start with the same concentration of oxyhæmoglobin, and to use the calibration

curve for this concentration. If the percentage of oxyhæmoglobin differs more than 10 p.c. from this, a corresponding, separate calibration curve is wanted. One experiment will now be described.

3 c.c. oxalate blood from the finger, containing 16·8 g. of hæmoglobin per 100 c.c. were delivered in a 50 c.c. volumetric flask, containing 0·15*N* Na_2HPO_4 and 1 p.c. of saponin, so that the total volume was 50 c.c. After 5 min. this solution was put into a very efficient small centrifuge, so that a very clear liquid was obtained. The 1000 c.p. Pointolite lamp had already been alight for 10 min. at 3·0 amp.; the drop in potential over the 10,000 ohm fixed resistance was 835 mv. with an accuracy of 0·5 mv. when the absorption cuvette was filled with water; the same drop with darkened selenium cell was 117 mv.

10 c.c. of the prepared solution were delivered in the 10 mm. absorption cuvette; the E.M.F. over 10,000 ohm was then 710 mv. Temperature was regulated by circulation of water around the circular, metal side-wall of the absorption cuvette; in this experiment the temperature in the cuvette during the reaction was $18 \pm 0{\cdot}04°$ C. 1 c.c. of 0·9*N* NaOH solution was injected into the filled cuvette by a calibrated tuberculin syringe, and well mixed by repeated suction and pressure. The stopwatch was started at this moment, and the decreasing E.M.F. over 10,000 ohm was determined every 30 sec. The potentiometer used was the new Cambridge electrometer valve potentiometer. Afterwards the reaction of the solution in the cuvette was determined by the bubbling hydrogen electrode at pH = 11·86.

Calibration.

After completion of denaturation in the cuvette the reaction was adjusted to about pH 8 by addition of 0·5 c.c. normal acetic acid. This may cause a precipitation of the denatured hæmoglobin, which re-dissolves at once when it is well mixed. A too gradual neutralization, for instance by CO_2 bubbles, may cause a true partial reversion of denaturation [Anson and Mirsky, 1931]. If one accounts for the dilution, caused by addition of 0·5 c.c. of acetic acid to 11 c.c. of globin hæmochromogen, the absorption in the cuvette has not changed by the partial neutralization.

Mixtures of the denaturated solution at pH 8 with the original solution of oxyhæmoglobin (diluted accordingly from 10 to 11·5) are now prepared by adjusting the amount of denatured hæmoglobin at pH 8 to a volume of exactly 10 c.c., and by exchanging 1 c.c. of it for 1 c.c. oxyhæmoglobin solution, and so on. The calibration curve, giving the relation of the percentage of unchanged oxyhæmoglobin in total pigment to drop in E.M.F.

over the 10,000 ohm resistance is seen in Fig. 2. The calculation of the rate of denaturation in percentages of unchanged oxyhæmoglobin from the observed drop in millivolts is made by this corresponding calibration curve.

Results.

The constant result of the examination of human oxyhæmoglobin of normal persons, aged 7–50 years, is the fact that the reaction first proceeds rapidly, then more slowly. If we make the assumption that there

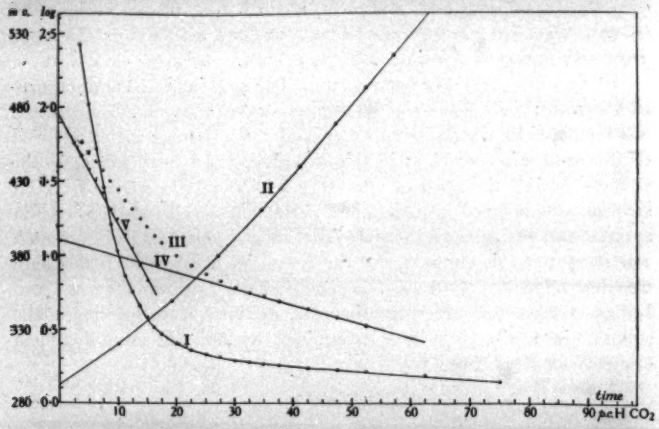

Fig. 2. Rate of alkali denaturation of the hæmoglobin of R. B., in 1·0 p.c. solution. Description in text, p. 384. I. Observed drop in millivolts. II. Calibration curve. III. Log percentage of unchanged C_1+C_2. IV. Extrapolation of C_2. V. Log C_1. Vertical axis, millivolts and log percentage unchanged hæmoglobin. Horizontal axis, time in minutes and percentage unchanged hæmoglobin (for II).

is no appreciable change in *p*H value, or other secondary reactions affecting the velocity constant during the course of the main reaction, the diminution in the reaction velocity may be attributed to the existence of a resistant form of hæmoglobin. Control experiments show that the change in *p*H is negligible. In an experiment similar to the one described, the *p*H was determined every minute by the bubbling hydrogen electrode; 1 min. after the addition of 0·9 *N* NaOH 1 c.c. *p*H was 11·83, 1 min. later 11·82, 2 min. later 11·82, 4 min. later 11·81, 6 min. later 11·80, 10 min. later 11·79, 15 min. later 11·79, 20 min. later 11·79.

TABLE I.

t min.	mv.	Percentage unchanged C_1+C_2	Log unchanged C_1+C_2	Extrapol. log C_2	Extrapol. C_2	By subtraction C_1	Log C_1	Calibration: Percentage unchanged hæmoglobin	Calibration: mv.
0	710·0	100·0	2·000	1·108	12·80	87·20	1·941	0	292
1·0	562·0	68·7	1·836	1·080	12·00	56·70	1·754	10·0	320
1·5	522·0	58·6	1·768	1·068	11·70	46·90	1·671	19·0	349
2·0	482·0	49·3	1·693	1·052	11·30	38·00	1·580	27·1	380
2·5	452·0	43·1	1·634	1·040	10·96	32·14	1·507	34·4	411
3·0	426·0	37·6	1·575	1·027	10·64	27·00	1·431	40·9	441
3·5	401·0	32·0	1·505	1·012	10·52	21·50	1·312	46·9	470
4·0	383·0	27·8	1·444	1·000	10·00	17·80	1·250	52·2	494
4·5	366·0	23·4	1·369	0·988	9·73	13·67	1·137	57·0	516
5·0	355·0	20·5	1·312	0·972	9·38	11·12	1·046		
5·5	345·0	17·7	1·248	0·960	9·12	8·58	0·934		
6·0	338·0	15·6	1·193	0·947	8·85	6·75	0·830		
6·5	331·0	13·5	1·130	0·932	8·55	4·95	0·695		
7·0	327·0	12·2	1·086	0·920	8·32	3·88	0·589		
8·0	320·0	10·0	1·000	0·891	7·78	2·22			
9·0	316·0	8·6	0·934	0·862	7·28	1·32			
10·0	313·0	7·55	0·878	0·838	6·89				
11·0	311·0	6·8	0·832	0·810					
12·0	309·0	6·1	0·785	0·785					
13·0	307·5	5·7	0·756						
14·0	306·5	5·35	0·728						
15·0	305·5	5·0	0·699						
17·0	304·0	4·4	0·644						
19·0	302·5	3·85	0·586						
21·0	301·2	3·35	0·525						
25·0	299·0	2·3	0·362						
30·0	296·0								
40·0	292·5								
50·0	292·0								

In Table I and Fig. 2 the result of the experiment described is analysed from this point of view. Calculations are made on the assumption that the hæmoglobin was composed of two kinds, with different rates of denaturation, the concentration of the type with the largest resistance against alkali denaturation being C_2, and of the other form C_1. Experimentally found is the amount of C_1+C_2 which is not denatured after a given time; the logarithms of these concentrations, plotted against time, show that from 12 min. after the beginning of the reaction the points fall on a straight line. This is interpreted as the monomolecular denaturation reaction of C_2, and extrapolation of log C_2 to $t=0$ gives 12·8 p.c. as the initial concentration of C_2. Subtraction of the extrapolated corresponding values of C_2 from C_1+C_2 gives the true rate of denaturation of C_1, which also seems to proceed as a monomolecular reaction.

In Fig. 3 a number of determinations on the hæmoglobin of R. B. are collected; *p*H and temperature during each experiment were constant but varied slightly in the separate examinations, as it is recorded in the legend. The denaturation velocity of C_1 is not designed here, but it was represented by a straight line in each case. Our interpretation is that the alkali denaturation of human oxyhæmoglobin is going on as two monomolecular reactions with different velocity constants. The values of these constants can of course be calculated, but the main object of this paper is the demonstration of the existence of two kinds of hæmoglobin in adult human blood. It is seen, that in the blood of R. B. the log of the concentration of the resistant form is found between 0·93 and 1·3, giving the amounts of resistant hæmoglobin as 8·5 to 20 p.c. of the total amount.

In the blood of other persons the results were quite similar, with a tendency in children to show higher values for the resistant form. The examination of the blood of the umbilical vein in new-born children is described by lines A and B in Fig. 3. In confirmation of former researches [von Krüger, 1927; Bischof, 1926; Haurowitz, 1929] the amount of resistant hæmoglobin in neonati is about 80 p.c. (similar determination in six cases).

Hæmoglobin of normal rabbit's blood, and the blood of horse, pig and ox, examined in the same way, but at *p*H 12·50–*p*H 13 has always given one straight line, indicating the presence of only one kind of hæmoglobin. How this may be altered in experimental anæmia is a subject under research now.

Checks have been made with known mixtures of hæmoglobin of adults and of new-born children. A typical example is given in Fig. 4. Here,

line I gives the rate of denaturation of adult hæmoglobin (pH 12·20, t 17·7° C.), line II the rate of denaturation of new-born's hæmoglobin at

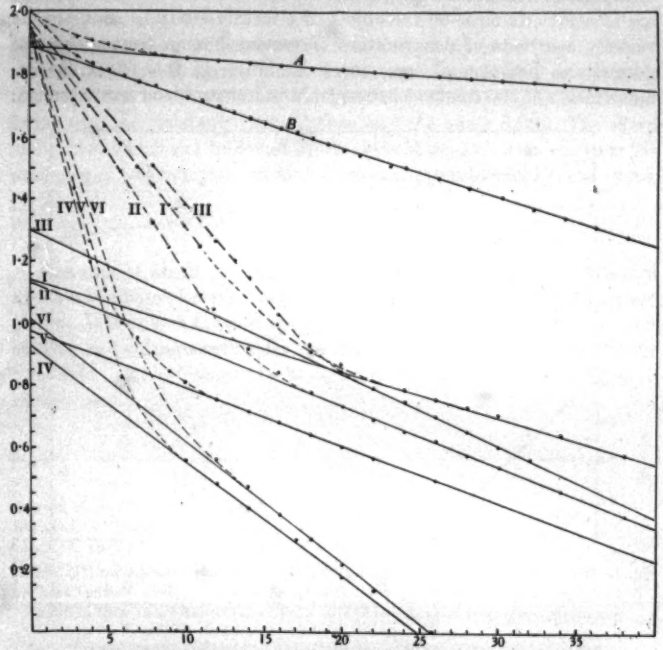

Fig. 3. Rate of alkali denaturation of the hæmoglobin of R. B., in 1·0 p.c. solution:

I. Sept. 4, 1933. pH = 11·80, t = 18·2° C.
II. „ 5, „ pH = 11·80, t = 18·0° C.
III. „ 10, „ pH = 11·80, t = 17·2° C.
IV. „ 14, „ pH = 11·86, t = 18·6° C.
V. „ 21, „ pH = 11·84, t = 18·5° C.
VI. „ 28, „ pH = 11·84, t = 18·8° C.

A. Hæmoglobin in 1·0 p.c. solution of new-born child pH = 12·00, t = 19° C.
B. Hæmoglobin in 1·0 p.c. solution of new-born child pH = 13·00, t = 19° C.

Vertical axis, log percentage unchanged hæmoglobin; horizontal axis, time in minutes.

the same pH and temperature, and line III the denaturation velocity of a mixture of 80 p.c. of I and 20 p.c. of II. It is seen that I indicates the presence of 7·1 p.c. of the resistant type, II gives 93 p.c. of this kind of

hæmoglobin and from III 19·2 p.c. is found; calculation from I and II gives 18·6 p.c. Further, it is seen that one cannot say that the resistant type of adult human hæmoglobin and the new-born's human hæmoglobin are identical. It must be the object of a further study to compare the velocity constants of denaturation of various human hæmoglobins of normal and pathological cases more carefully. In this paper the demonstration of two kinds of hæmoglobin in human blood was intended.

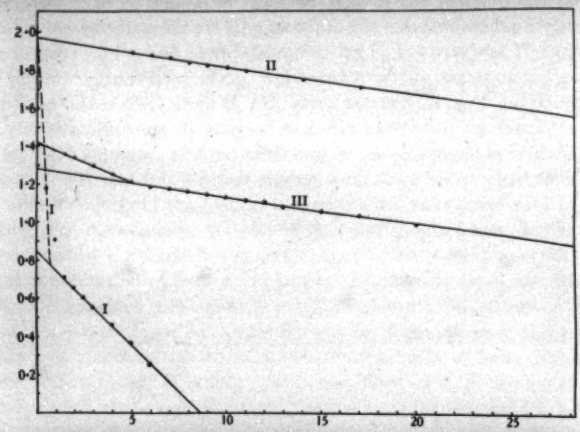

Fig. 4. Denaturation rates of adult hæmoglobin (I), new-born's hæmoglobin (II), and of a mixture of 80 p.c. I and 20 p.c. II. Description in text (p. 385). Vertical axis, log percentage unchanged hæmoglobin in total mixture; horizontal axis, time in minutes.

Discussion.

It appears to be generally accepted that, if an eventual difference in various hæmoglobins is present, this specificity must be located in the globin part of the molecule. Evidence for this supposition is growing continuously; apart from the physico-chemical methods of difference in absorption spectra, of crystal form, of solubility and of differences in isoelectric zone and rate of alkaline denaturation, we have the direct chemical analyses of globin. The researches of Schenck [1930] demonstrate a constant difference in the amount of certain amino-acids in the globins of various species. In human blood, especially, the arginine content of globin is constantly higher in the blood of new-born children than

in adult blood. In hypochrome cases of anæmia with normal regeneration this juvenile type seems also to be present; in pernicious anæmia it is missing.

Schenck also shows that the chemical differences of human globins correspond to their different resistances to hydrolysis by pepsin HCl.

Unsolved is the question whether human hæmoglobin is changing from one form to the other, or whether there are two, originally different forms (*e.g.* the resistant "fœtal" type and the adult form). The sharp distinction which can be found in the rates of denaturation suggests the existence of two independent kinds, not connected by transitional types.

Summary.

The rate of alkali denaturation of hæmoglobin, studied by means of a sensitive photo-electric arrangement, was determined in human hæmoglobin. It is shown that, in normal blood, alkaline denaturation is proceeding as two monomolecular reactions with greatly differing velocity constants. This indicates the presence of two kinds of hæmoglobin in human blood.

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SOME FORMS OF APPARATUS FOR THE EQUILIBRATION OF BLOOD.

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DILL in the appendix to *Blood* by L. J. Henderson [1928] described an excellent technique for the equilibration of blood and its transfer to the van Slyke manometric apparatus. He says, "We propose to describe a technique which we have used when as much as 135 c.c. of blood... were available." The methods described below make no profession of improving upon the accuracy of that technique; their aim has been, while yielding as little as possible in the matter of accuracy, to use very much less blood. The van Slyke manometric apparatus analyses 1 c.c. or even 0·2 c.c. of blood with great accuracy, but the whole process of equilibration, involving the use of large dead spaces, etc., demands, for the determination of dissociation curves, quantities of blood of a much higher order—quantities that are often quite unattainable.

The apparatus used requires little description; several forms have been employed, according to the amount of blood available.

I. For duplicate analyses of 1 c.c. samples.

The apparatus is shown in Fig. 1. The main saturating vessel *A* is of about 350 c.c. capacity. It is of about 25 cm. length from *M* to *G*. The stopper is of glass and is of special design. It consists of three concentric tubes. The outermost is the stopper proper. The cavity between this and the middle tube communicates with the air by a three-way tap *F*. This tap is for the injection of the blood. The middle tube is produced about 1½–2 cm. (to *D*) further into the saturator than the stopper. It ends in a flange, otherwise blood may find its way within this tube—which is to be avoided. The bore of the stopper at *G* is about 2 cm., that of the tube *D* about 1 cm. The innermost tube is really the "van Slyke pipette." The cavity between this and the tube *D* com-

municates with the air by another three-way tap *E*. This is for the abstraction of gas samples. If blood never gets into the tube *D* there is no chance of fouling the Haldane gas analysis apparatus. The van Slyke pipette is made of 1 mm. tubing, as also are the three-way taps. It has two bulbs *X* and *Y*, each of 1 c.c. between the graduation marks. The tube reaches, within the saturator, as nearly as possible to *M*, where it is narrowed somewhat at the tip. In the other direction after traversing the stopper, it possesses the usual tap *C*, beyond which it is produced far enough to deliver blood properly into the van Slyke apparatus.

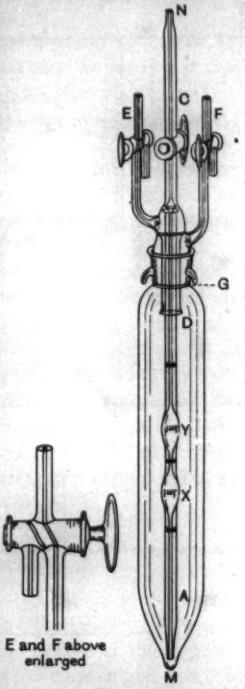

Fig. 1.

The saturator, after evacuation, is filled in the usual way from gas reservoirs, after which it should have a positive pressure of a millimetre or two. It is then placed in water at laboratory temperature and one of the taps opened momentarily so that the gas is reduced to atmospheric pressure at a known temperature, say 15° C. Blood is injected through tap *F*—3½ c.c. should be sufficient. The dead space is cleared with mercury or nitrogen; the latter is better, as there is then no risk of mercury entering the saturator—frequently air will serve. Equilibration then takes place by rotation in the horizontal position (see description of bath later), after which the saturator, still in the equilibration tank, is placed vertically with *M* downwards. If, since the introduction of the blood, it has never been in this position before, no blood can have entered the pipette or soiled the end. The tank, Fig. 2, *A*, must be deep enough for the saturator to remain completely immersed (apart from the projecting end of the pipette). When sufficient time has been allowed for drainage the tap, Fig. 1, *C*, is very cautiously opened. The positive pressure (the saturator is usually equilibrated at a higher temperature than that at which it was filled) forces the blood up into the pipette. When this is quite full the saturator is removed from the bath, inverted, and the blood

transferred to the gas analysis apparatus without opening the saturator. In practice there is no difficulty in seeing the graduations of the pipette through the walls of the saturator. The only operation which requires a word of caution is the filling of the pipette. It is necessary to open the tap very gradually and to watch the progress of the blood as it fills

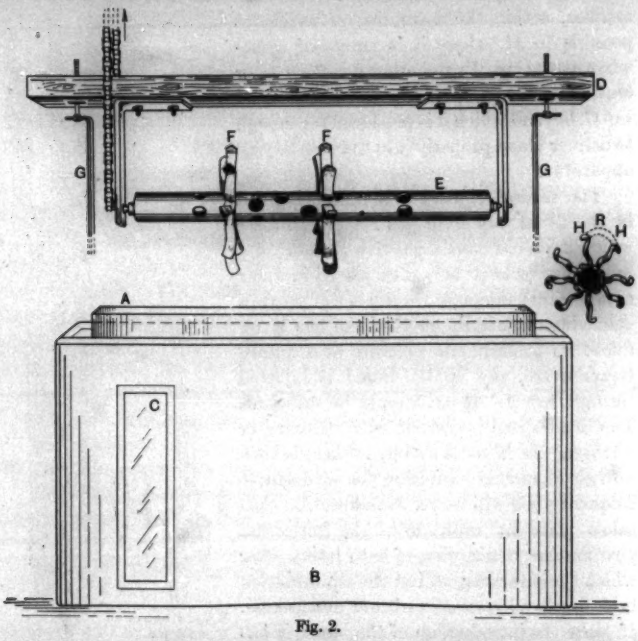

Fig. 2.

the pipette. If the blood is allowed to go in at all rapidly, especially in the smaller types of saturator to be described later, vortices of air form in the blood, and a froth appears in the pipette.

Bath.

The bath which we use is of the following design. The actual bath is a standard sized glass tank, Fig. 2, *A*, 53 × 34 × 34 cm. approximate inside measurement. This is placed in a wooden lidless box (*B*) which is

fitted with a drainage tube. The inside of the box is about one inch longer and broader than the outside of the glass tank.

Into the wood near one corner is let a window *C*, about 5 cm. broad and 25 cm. high. The saturator when in the vertical position is held in a clip attached to the glass and can be viewed through the window, so that the blood can be seen entering the pipette.

The spindle *E* on which the saturators are mounted during rotation is suspended from a stout piece of wood *D*, which rests on the top of the bath and is kept in position by a strap *G*, of sheet brass, passing round the bath. For the sake of clearness, it is shown in Fig. 2 *above* the bath, not in it.

The saturator is held in its place by spring clips, *F*, *F*. End on they are shown as *H*, *H*. The saturator is kept firmly in position by a rubber band *R*. The clips are sufficiently flexible to serve for saturators of different calibre (see below), the grip being regulated by the strength of the rubber band. String may be used.

With the bath placed inside the wooden box, the temperature can be regulated accurately enough for many purposes, by the use of an electric candle lying near the bottom of the bath fitted with a resistance outside; but of course, for accuracy over a long time a thermostat is preferable. Apart from cases in which the oxygen pressure is very low, 10 min. equilibration should suffice. In the observations necessary for ordinary oxygen dissociation curves 15 min. is long enough in all cases.

The following are five sets of duplicate analyses of the percentage saturation of hæmoglobin exposed to various oxygen pressures taken at random:

(1)	(2)	(3)	(4)	(5)
40·9	49·5	57·2	59·5	73·5
41·4	48·7	57·9	59·9	73·6

There seems to be no certain tendency for the top bulb to give a higher reading than the bottom one or *vice versa*. It had been feared that owing to the formation of dew in the bulbs the first sample of hæmoglobin solution to emerge from the pipette might be the more dilute, but this does not seem to be appreciable.

II. Smaller forms of apparatus.

The saturators have been made in smaller sizes. For many purposes an apparatus of about 60 c.c. capacity has proved useful. This does not differ in principle from that described. The stopper is of the same dimensions, the taps, etc., are of rather narrower bore tubing and the bulbs of the pipette are each 0·2 c.c. capacity. The saturator from *G* to *M* is

about 11 cm. long. The pipette measures from the tip. There is no dead space. Otherwise Fig. 1 sufficiently describes it. A still smaller size, Fig. 3, has been used, of about 25–30 c.c. capacity. This demands a smaller

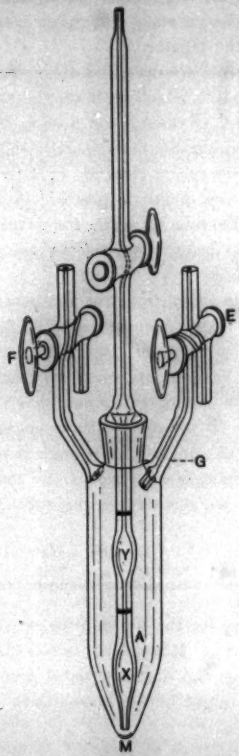

Fig. 3.

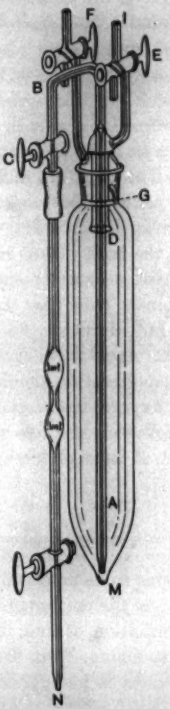

Fig. 4.

stopper and therefore a different design of apparatus, about which there is little that does not appear in Fig. 3. The bulbs are of 0·2 c.c. and the tubing from taps *E* and *F* is attached to the chamber not to the stopper, and the gas tube (connected with *E*) is continued about half a centimetre or more inside the saturator.

The following figures give the percentage saturation of five samples of blood exposed to different oxygen tensions—they are taken at random:

(1)	(2)	(3)	(4)	(5)
22·5	52·6	80·0	87·0	83·3
22·3	52·4	81·8	87·0	83·7

III. Alternative form of saturator.

Another form of saturator which gives equally accurate results is shown in Fig. 4. It was devised earlier than that shown in Fig. 1 and, though we do not now use it, there are two reasons for mentioning it. Firstly, researches were carried out with it which will be published later, and secondly, while we consider the balance of advantage to be against it, others may not take that view. The essential difference is that the measuring pipette is outside the saturator vessel to which it is attached by pressure tubing. The drawbacks are: (1) that the pipette and the attached tubing down to the orifice below *A* are filled with mercury; (2) there is the danger of a bubble of air being trapped in the rubber tubing and getting into the pipette with the blood; (3) rather more blood is necessary than in the form shown in Fig. 1. When the blood has been equilibrated and allowed to pass into the pipette, the rubber tube is cut and the pipette removed. The advantages are: (1) that the pipette is more easily read when the blood is passing into the van Slyke apparatus, and (2) that the apparatus is less easily broken.

Summary.

Forms of saturator are described, which aim at the attainment of considerable accuracy, with a less quantity of blood than is used by accurate methods already in use.

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612.744.211

THE EFFECT OF DURATION OF WORK ON THE EFFICIENCY OF MUSCULAR WORK IN MAN.

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Investigations on the influence of the time factor on the efficiency of human muscular work have proceeded on two distinct, though by no means independent, lines, namely, the determination of the effects of rate of working and of duration of performance. Many of the early investigations showed that the rate of working materially influences the efficiency of performance, and it is therefore essential to pay due regard to this in any experimental study on the effect of duration of work on the mechanical efficiency of man which is the subject of the present investigation.

In Benedict and Cathcart's [1913] bicycle ergometer experiments with the professional cyclist subject M.A.M., the net efficiency (*i.e.* the percentage ratio of external work performed to the increased energy expenditure indicated by the oxygen used for work in excess of the resting consumption of the subject when seated on the ergometer) was found to vary with the number of pedal revolutions per minute. The maximum efficiency was attained at approximately 70 pedal revolutions per minute, but the efficiencies at slower rates were not fully investigated. It should be noted that each pedal revolution involves two complete leg movements so that at 70 revolutions per minute the contraction-relaxation time per leg movement for each leg would be 0·86 sec., although the actual duration of contraction would probably be 0·43 sec. or less, as pointed out by Garry and Wishart [1931].

Hill [1922] showed that the actual rate of change of muscle form, or speed of shortening of the muscle, is a factor influencing the energy expended in contraction, and Dickinson [1929] published an account of experiments on the efficiency of pedalling a bicycle ergometer at

varying speeds. Dickinson found that for work of short duration the excess oxygen used in work and recovery indicated a maximum net efficiency of performance when the rate was 33 pedal revolutions per minute, *i.e.* 1·82 sec. per contraction-relaxation time or approximately 0·9 sec. per contraction of the effector muscles.

Table I, compiled from Dickinson's figures for the experiments in which the load was practically constant, gives some idea of the variations in efficiency which were observed at different rates of pedalling and it may be pointed out that between 25 and 48 pedal revolutions per minute, *i.e.* when 1·2–0·63 sec. was allowed for contraction, there was relatively little variation in efficiency. Outside this range, however, the efficiency materially decreased.

TABLE I. Results of Dickinson's experiments with constant load and varying rates of pedalling bicycle ergometer.

Load (kg.)	Pedal revolutions per min.	Contraction-relaxation time (sec.)	Approximate contraction time (sec.)	Net efficiency p.c.
3·0	111	0·54	0·27	11·4
3·2	60	1·00	0·5	18·7
3·1	48	1·26	0·63	20·5
3·2	38	1·56	0·78	20·1
3·4	33	1·80	0·90	21·8
3·0	33	1·80	0·90	21·5
3·05	25	2·40	1·20	20·0
3·3	11	5·42	2·71	15·1
3·2	8	7·24	3·62	13·4

Garry and Wishart [1931] repeated some of the earlier experiments of Benedict and Cathcart in order to examine more closely the efficiencies of pedalling at the slower rates, namely below 70 pedal revolutions per minute. Their experiments differed from Dickinson's in that the excess oxygen consumption for the work was observed during the "steady state," 25 min. previous pedalling at the rate under investigation being performed before the collections of expired air were made. They found that a maximum efficiency was attained at 52 pedal revolutions per minute, namely when the contraction-relaxation time for the effector muscles was 1·15 sec., which allowed not more than 0·6 sec. for the actual duration of contraction. The optimum gross efficiencies observed were 16·3 and 18 p.c., while the net efficiencies were 2–3 p.c. higher.

Common experience teaches that the height of the saddle above the pedals must be carefully adjusted to suit the leg length of the individual if the effort of cycling is to be carried out with maximum comfort and

ease; moreover, it is not unlikely that the weight and inertia of the legs of a subject together with the linear length of the pedal stroke in relation to leg measurements and the position of the saddle in relation to the pedals may affect the optimum rate of pedalling. Again, unless the gearing and load were the same from ergometer to ergometer, the work done in starting the machine and per pedal revolution after speed had been attained would be widely different and the actual effective load per pedal revolution cause the efficiency to vary. If one could be certain that the whole of each effector muscle were in contraction for the complete duration of the down stroke in each leg movement, then perhaps more faith could be placed in comparative studies of the data obtained by different observers. However, in general terms it is quite clear that the results of investigations on human muscular efficiency all point to the conclusion that the rate of change of muscle form, or speed of contraction of the muscles, profoundly affects the efficiency of performance, and, therefore, that due regard to this should be paid in all studies of energy expenditure in muscular work when that work is executed at different rates and in varying ways.

Simonson and Hebestreit [1930] published an account of experiments on the mechanical efficiency of the human body in relation to the duration of work, and claimed to have shown that the efficiency of a man for short spells of work, *e.g.* of 1 min., is very much less than when he performs the same amount of work per minute for 3, 6 or 10 min. In some cases the efficiency was twice as great in the 10 min. spell of work as compared with the 1 min. as shown in Table II, which gives some of their results calculated in terms of percentage efficiency instead of calories per kilogram-metre.

TABLE II. Results of efficiency experiments on a horizontal pull ergometer with varying duration of work. (Calculated from the data of Simonson and Hebestreit [1930].)

Load and rate of working	Duration of work and efficiency p.c.			
	1 min.	3 min.	6 min.	10 min.
Load 10 kg. 20 pulls per min.	7·0	9·7	10·0	10·5
	6·3	11·4	12·5	13·2
	9·7	11·3	11·4	13·9
Load 10 kg. 30 pulls per min.	9·4	9·8	11·3	13·2

In their experiments, muscular work of two kinds was studied: (*a*) on an ergometer in which the subject exerted a simple horizontal pull by means of the arm muscles while standing or sitting with the spine vertical, and (*b*) stair climbing at agreed rates per minute. In both cases it would

be easy for the subject to execute the requisite number of muscular movements sharply in the minute spells and adopt a steady slower rate of muscular shortening for the long spells of work while executing the same number of pulls, or ascending the same number of stairs, per minute in each case. It is not clear from their records that the same rate of change of muscle form was ensured in the long spells of work as in the short ones.

Apart from drawing conclusions as to the significance of their results in relation to muscular work by man, Simonson and Hebestreit claimed that the changes in efficiency with prolongation of work which they observed indicated that the chemical reactions attendant on muscular contraction also changed and were therefore more complex than those put forward by Hill and others in the current theories of muscular contraction based on studies of isolated muscles. They claimed therefore that their results proved the inapplicability to man of knowledge gained by the study of isolated muscle.

Were the experiments and deductions of Simonson and Hebestreit fundamentally sound, not only would the current theories of the energetics of muscular contraction be shaken, but their results would be of considerable significance in studies of muscular work in industry. It was therefore of importance that experiments should be carried out with all possible safeguards in order to ascertain whether in actual fact fully loaded human muscles working at the same rate per minute and shortening in the same time per contraction did actually perform external work with less energy expenditure and increased efficiency in long spells of work (6 or 10 min.) as compared with similar efforts exerted in spells of short duration (1 min. or less). The following experiments were therefore devised and carried out in order to ascertain whether Simonson and Hebestreit's results could be obtained under such conditions.

Experimental procedure.

The experiments were designed so that the rate of working, the speed of shortening of the effector muscles, the total amount of work done and the load opposing the effector muscles per contraction should be the same whether the work was performed in short spells (intermittent work) or in long spells (continuous work). Moreover, the load was such that it would involve a maximal effort on the part of the effector muscles of the subject. The type of work selected was pedalling a Martin's bicycle ergometer, as this permitted accurate control of the various factors mentioned above. The attachment of a revolution counter with a loud

click to the back wheel of the ergometer enabled the subject easily to adjust and maintain his rate of pedalling so that the ticks of the metronome synchronized with the clicks of the counter.

A young man, H. L., who was a skilled cyclist and accustomed to metabolism experiments, was selected as subject. His data and the measurements of the bicycle ergometer when adjusted to his stature were as follows:

Subject H. L. Age 20.
Height 165 cm.
Weight 57·2 kg.
Total leg-length 75 cm.
Length knee to ground 43 cm.

Bicycle ergometer: Mid-saddle to pedal maximum 82 cm.
Mid-saddle to pedal minimum 47 cm.
Circumference of back wheel 144 cm.
Gear ratio 3 : 1.

As a routine the subject started resting, sitting 2 hours after a light breakfast and after 30 min. took up the work position on the ergometer. After a further period of rest in the work position a 10 min. collection of expired air was taken. The work spells, three of which were carried out each experimental session, were arranged to alternate as follows: intermittent work, continuous work, intermittent work; or continuous work, intermittent work, continuous work. Throughout each session the subject remained seated on the ergometer, and following the recovery collection, after each working effort, a resting collection of expired air was made.

The intermittent work consisted of ten short spells of 25 sec. pedalling effort, each spell being followed by rest up to 3 min. so that 250 sec. actual working time was evenly distributed over a period of 30 min. A further 15 min. was allowed for final recovery, the total expired air for 45 min. being collected in a 1000 litre Douglas bag.

The continuous work spell consisted of 250 sec. pedalling at the same rate per minute and speed of contraction as during the short spells followed by recovery up to 45 min., the whole of the expired air in work and recovery being collected. At the end of each session a final 10 min. resting collection of expired air was made while the subject was still in the work position on the ergometer.

It may be pointed out that the total oxygen cost of the effort during work and recovery must be ascertained in a study such as this, and as

the attainment of the "steady state" is impossible the whole of the expired air during work and recovery has to be collected in each case.

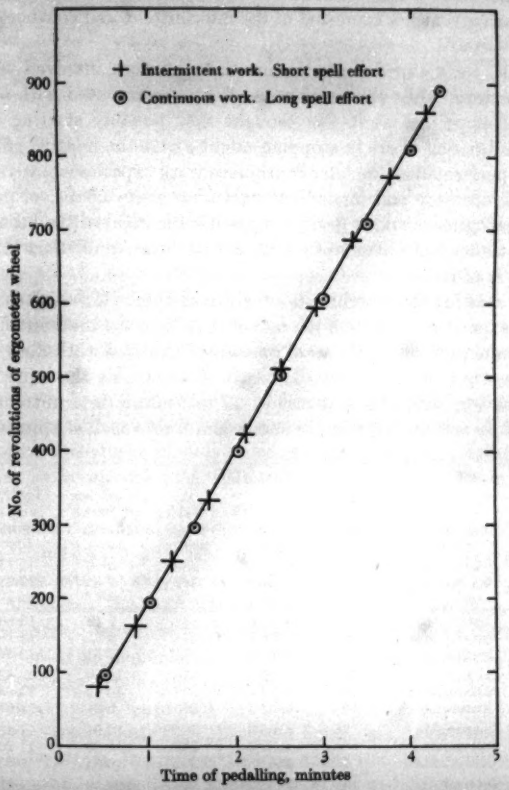

Fig. 1. Chart showing number of revolutions of bicycle ergometer wheel in ten bouts of 25 sec. effort (short-spell intermittent work), and number of revolutions observed at intervals of 30 sec. during the long-spell continuous effort of 250 sec. duration. 14. v. 31.

After every 25 sec. pedalling in the intermittent work and also during the continuous effort the number of revolutions on the counter were noted. At frequent intervals the minor variations in the spring balance attached

to the ergometer were noted, these being averaged for each experiment in order more accurately to evaluate the effective friction load due to the suspended weight. Fig. 1 shows how closely the rate of execution of the muscular movements coincided in the intermittent and continuous work spells.

In the early experiments the intermittent work involved ten starts and ten stops, while the continuous effort was executed with one start and one stop; and as it was thought that possibly starting and the negative braking effort in stopping might affect the relative efficiencies of total performance, the later continuous work experiments were carried out with one stop and immediate start after every 25 sec. of pedalling, thus making the work as nearly as possible identical in the intermittent and continuous efforts except for the actual duration of effort in relation to periods of rest.

The data for the experiments are given in Table III, which shows that in each experimental session the rate of pedalling, the total number of leg movements performed, the total amount of external work done and the work per leg movement, were as nearly as attainable the same whether the work was executed in bouts of 25 sec. effort (intermittent work), followed by rest up to 3 min., or in one continuous spell of approximately 4 min. duration.

TABLE III.

Experiment date and order	Character of work	No. of starts and stops	Total work done kg.m.	Total leg movements	Work per leg movement kg.m.	Total O_2 cost of work litres	Net efficiency p.c.
11. v. 31 i	Intermittent	10	5481·8	556	9·86	12·735	20·19
ii	Continuous	1	5426·2	562	9·65	14·985	16·99
iii	Intermittent	10	5339·4	558	9·57	14·467	17·32
14. v. 31 i	Continuous	1	5342·8	582	9·18	13·680	18·33
ii	Intermittent	10	5368·5	574	9·35	12·195	20·50
iii	Continuous	1	5529·9	594	9·31	13·860	18·73
27. v. 31 i	Continuous	10	5760·0	576	10·00	15·525	17·42
ii	Intermittent	10	5814·3	574	10·13	14·850	18·72
iii	Continuous	10	5822·5	586	9·94	14·670	18·62
28. v. 31 i	Continuous	7	4166·9	382	10·91	11·430	17·11
ii	Intermittent	7	4332·9	392	11·05	11·205	18·15
iii	Continuous	7	4057·8	372	10·91	9·630	19·77
29. v. 31 i	Intermittent	10	5355·7	570	9·39	11·520	21·82
ii	Continuous	10	5335·4	562	9·49	12·780	19·59
iii	Intermittent	10	5474·3	576	9·50	11·950	21·50

The rate of pedalling was 72 revs./min. throughout.

Table IV gives the net efficiencies calculated from the data in Table III and set out in order of performance of the effort whether intermittent or continuous. It is clear that whether the work done was

executed in short spells or in long spells there was little difference in the efficiency of performance.

TABLE IV. Net efficiencies in short- and long-spell work.

Short-spell intermittent work	Long-spell continuous work	Short-spell intermittent work	Long-spell continuous work
20·19	16·99	17·32	
	18·33	20·50	18·73
	17·42	18·72	18·62
	17·11	18·15	19·77
21·82	19·59	21·50	

The mean efficiency for the intermittent short-spell work was 19·74 p.c. as compared with a mean efficiency of 18·32 p.c. for the same amount of work when executed at the same rate of muscular movement in one continuous effort. Although the difference between the mean efficiencies cannot be regarded as significant, yet it is worthy of note that when the subject executed the work in spells of short duration his net efficiency was on the whole slightly higher than when the same total amount of work was performed in one long spell. These experimental results are, therefore entirely at variance with the general claim made by Simonson and Hebestreit that their experiments proved that the efficiency of performance was approximately twice as great in long-spell effort lasting 6–10 min. as compared with that attained in work of 1 min. or less duration.

As shown in Table I above, Dickinson obtained efficiencies varying from 11·4 p.c. at a maximum rate of pedalling to 21·8 p.c. with an optimum rate, namely an order of difference approximating to the results of Simonson and Hebestreit. Further experiments were therefore carried out in order to ascertain whether such a variation in efficiency could be demonstrated when the subject performed the same total amount of work in short-spell bouts at a maximum rate and in long-spell work at an optimum rate.

In these experiments the subject did 6 min. work at a rate of 0·9 sec. per leg movement—this being the long spell; the short-spell effort was for 6 min., each minute being made up of 20 sec. pedalling at a maximum rate of 0·29 sec. per leg movement followed by rest up to 1 min.

As shown in Table V the efficiency of performance during the short-spell effort was from 5 to 7 p.c. less than that attained during the long spell.

It is apparent from the data given in Table V, and from the well-known influence of variations in the speed of muscular movement on

TABLE V.

	Long-spell continuous work at optimum rate: 0·9 sec. per leg movement	Short-spell intermittent work at maximum rate: 0·29 sec. per leg movement	Long-spell continuous work at optimum rate: 0·9 sec. per leg movement
No. of starts and stops	6	6	6
Rate of pedalling: revs./min.	33	101	33
Total work done: kg.m.	3571·5	3822·5	3758·5
Total leg movements	382	406	404
Work per leg movement: kg.m.	9·35	9·41	9·30
Total O_2 cost of work: litres	7·530	11·580	8·370
Net efficiency p.c.	22·25	15·51	21·07

efficiency, that the results of Simonson and Hebestreit may be, in part, accounted for by more rapid change in muscle form in work of short duration than in long-spell work.

Hill [1932] drew attention to this possible explanation of their results in 1931, and since that date Simonson [1933] has re-examined his data and carried out further experiments in which he claims to have substantiated his former results and to have proved that variations in speed of movement could not account for the changes in efficiency which he has observed. In the later experiments Simonson and Sirkina [1933] employed a different type of effort, namely lifting dumb-bells varying in weight from 2 to 8 kg. In these experiments the subject first stood with arms by the side and then lifted the dumb-bell with extended arm until level with the shoulder. The lifting and lowering efforts were carried out at the same speed. Rates of 10 or 20 lifts per minute were studied and the duration of effort varied from ½ to 7 min. With this type of effort and with the lightest loads he found wide variations in efficiency in relation to the duration of the work. In some cases the efficiency attained in the 7 min. spell was nearly 6 times as great as that for the ½ min. spell of similar work. Simonson gives his results in terms of small calories energy expended per kilogram-metre of work done, but this method of presentation obscures the fact that the type of work he selected was performed by his subjects at remarkably low efficiencies as shown in Table VI which gives some of his data and the actual efficiencies as calculated from them.

It is well established that purely dynamic muscular effort in man can be performed with an efficiency of the order of 25 p.c., but if the character of the work involves postural strain or static effort, then the expenditure of energy per unit of external work as measured is proportionately increased and the efficiency of man as a whole is lessened. Such low efficiencies as 0·83, 1·98, or 4·93 p.c. are probably due to a relatively large

TABLE VI. Efficiencies for dumb-bell lifting. (Calculated from Simonson's data [1933].)

Load kg.	Lifts per min.	¼ min. spell cal./kg.m.	¼ min. spell Efficiency p.c.	7 min. spell cal./kg.m.	7 min. spell Efficiency p.c.	Efficiency ratio ¼ min./7 min.
2	10	280	0·83	47·5	4·93	5·9
4	10	202	1·16	38·6	6·07	5·24
6	10	118	1·98	38·3	6·12	3·08

expenditure of energy by static effort of some kind, and it is of interest to note that when Simonson employed heavier dumb-bells, or increased the number of lifts per minute, he did not get such remarkable changes in efficiency in relation to the duration of the effort, and that the efficiency in the initial short-spell effort was higher.

Simonson's figures show that load and speed of movement in work of this character affect the efficiency profoundly. For example, with a 6 kg. dumb-bell lifted in 0·75 sec. and lowered in 0·75 sec., for a period of ¼ min. (10 movements), the efficiency was 4·17 p.c., but with a 2 kg. dumb-bell lifted in 3 sec. and lowered in 3 sec. for a period of ¼ min. (five movements) the efficiency was only 0·83 p.c., *i.e.* one-fifth of the former efficiency although the duration of work was the same in both cases.

It is evident that Simonson's data, on the basis of which he claims to have established a two to sixfold increase in efficiency in work of 7 min. duration as compared with the efficiency for work lasting ¼ min., were obtained using a type of work involving postural strain, static effort, submaximal loads and the execution of the muscular movements at suboptimal rates. Moreover, the increased efficiency ratio is calculated from initial experiments in which the expenditure of energy on dynamic muscular effort was very small indeed.

In discussing the method and results of the bicycle ergometer experiments described above as briefly referred to by Hill [1932], Simonson [1933] not only raises objection to the use of this ergometer but states that the time interval of rest up to 3 min. allowed for recovery after each 25 sec. pedalling was insufficient, and that to obtain his results 30 min. should be allowed between successive bouts of short-spell effort. It is quite true that in the bicycle ergometer experiments a number of short spells of work were carried out instead of one short spell only as employed by Simonson, but this modification of experimental routine was introduced for two reasons, namely to ensure that there would be a reasonably large increase in oxygen consumption

due to the work, and to enable the same total amount of work to be executed in the short-spell bouts as a group as in the long-spell effort.

In view of Simonson's contentions further experiments on the bicycle ergometer have been carried out and every effort has been made to obtain his results by using submaximal loads, very slow rates of pedalling and a single short-spell effort of 1 min. The same subject was used as for the earlier experiments. The whole of the expired air during work and recovery for the 1 min. and 6 min. spells was collected as before, but in the case of the short-spell effort two Douglas bags were used, the expired air for the 1 min. work and 3 min. recovery being collected in one bag while the second bag was used for 26 min. further recovery.

It will be seen from the data of these experiments as shown in Table VII that Simonson's results were not confirmed, for the mean efficiency for work of 1 min. duration was 17·86 p.c. as compared with 18·19 p.c. for precisely the same type of effort when continued for 6 min.

TABLE VII. Efficiencies for short- and long-spell work on the bicycle ergometer.

	Short-spell work 1 min.	Long-spell work 6 min.	Short-spell work 1 min.
No. of starts and stops	1	1	1
Rate of pedalling: revs./min.	20	20	20
Total leg movements	40	240	40
Total work done: kg.m.	152·9	912·38	153·79
Work per leg movement: kg.m.	3·82	3·8	3·84
Total O_2 cost of work: c.c.	442	2165	370
Net efficiency p.c.	16·23	18·19	19·5

It should be noted that in the experiments detailed in Table VII the total work done in 1 and 6 min. respectively closely corresponded in amount to that done by Simonson's subjects in the experiments on the horizontal pull ergometer, Table II above; but whereas Simonson's 1 min. spell showed an efficiency of 6·3 p.c. an efficiency of 16·23 p.c. was attained on the bicycle ergometer. It is possible, therefore, that static and postural components in Simonson's effort may partly account for his results.

DISCUSSION.

In all the experiments which have been carried out on the bicycle ergometer in which uniformity has been ensured in load, rate of working and speed of muscular movement, no material difference in efficiency has been observed whether the work was performed in short spells of 1 min. duration or less, or in spells of work of 6 min. duration.

The results of this investigation do not support the conclusions of Simonson and Hebestreit who claim to have shown by experiments on a horizontal pull ergometer and dumb-bell lifting that a two to sixfold increase in efficiency occurs in man when the duration of muscular effort lasts for 6 or 10 min. as compared with similar work executed for ¼ or 1 min. Simonson contends that the type of effort involved in pedalling a bicycle ergometer is not suitable for demonstrating his results, but it is difficult to understand why, if his phenomenon is, as he claims, a fundamental characteristic of muscular work in man, it should not be demonstrable on a machine such as the bicycle ergometer on which the additional effort occasioned by the work is mainly dynamic and expended by the effector muscles themselves with almost a minimum of postural strain. It would appear from what has already been said that the type of work selected by Simonson is not suitable for the study of the fundamentals of dynamic muscular effort in man, and therefore that the results of his experiments do not constitute sufficient justification for him to advance criticisms of existing theories of the intimate metabolic processes involved in muscular contraction, or to make generalizations in regard to the effect of duration of work on efficiency.

Although it is necessary to criticize Simonson's deductions on these grounds it is not thereby intended to throw any doubt on the accuracy of his experimentally determined data, for if his results are considered in the light of recent work on muscle it is apparent that they are precisely what would be predicted as likely to be found in experiments of the nature carried out by him.

Adrian and Bronk [1928, 1929] showed that increasing strength of contraction (lifting the heavier dumb-bells in Simonson's experiments) is the result of increased frequency of motor impulses leading to a fused tetanus together with the employment of a larger number of neuro-muscular elements in some cases. Bronk [1930] showed by a series of experiments on the sartorius of the frog that it is possible for a muscle to exert a continued force more economically if separate muscle twitches give place to a fused tetanic contraction, and that the economy in energy expenditure increases with the degree of fusion of the twitches until complete fusion is reached. The increase in economy, or in the efficiency of production of "tension-time" amounted to three or fivefold with increasing fusion of twitches due to increased frequency of stimulation, and it is of interest to consider this result in connection with Simonson's experiments, quoted above, in which in the case of a light load (2 kg. dumb-bell lifted slowly) the efficiency was 0·83 p.c., while for

a 6 kg. dumb-bell lifted four times as rapidly but for the same duration of work, ¼ min., the efficiency was 4·17 p.c., *i.e.* five times as great.

However, the main point at issue in the present discussion is the variation in efficiency in relation to duration of effort, and here again a clue to the explanation of Simonson's two to sixfold increase in efficiency is to be found in the recent studies on the heat production and economy of energy expenditure in maintaining muscle contraction. Bronk [1930] recalled the fact that the earlier work of Hartree and Hill [1921] indicated that the heat production at the commencement of a contraction is relatively greater than at later periods during its maintenance. From the point of view of the economy of production of tension time in relation to duration of stimulation Bronk investigated the well-known characteristic of muscle that prolonged stimulation at a uniform frequency initially below that required to produce summation leads finally to a complete tetanus. Working with the sartorius of the frog he found that with the increasing fusion of twitches as the stimulus was prolonged the economy in maintaining muscular tension also increased until at the end of a 2 min. period of stimulation at a frequency of 4 per second more than a fivefold increase in economy was recorded.

Bozler [1930] working with the retractor muscle of the pharynx of the edible snail obtained results similar to those of Bronk and showed that the economy of maintaining tension in long contractions increases six to ten or even fifteenfold following a few minutes' moderate exercise. More recently Bronk [1932] carried out experiments on crustacean muscle, and with the adductor muscle of the crab proved that the increase in economy of maintaining contractions is due to the slowing of the muscle as a result of previous contractions. This slowing of the muscle and prolongation of relaxation time permits summation and fusion of twitches in spite of a very low frequency of motor impulses, and with increasing fusion of twitches the economy of maintaining the contraction increases four or fivefold.

The results of these investigations have an important bearing on problems concerning the maintenance of postural tone and the prolonged contractions involved in static effort. On the other hand, in rapid dynamic effort, involving little postural strain or static effort, as in the case of rapid pedalling on the bicycle ergometer, the changes produced by prolonged activity in the time relations of contraction would have little influence, except on the optimum speed. Inasmuch as the type of work studied by Simonson involved a large—indeed a predominant—static component, ample opportunity was afforded for the slowing of muscle

contractions resulting from previous activity to increase the efficiency. It seems reasonable therefore to ascribe the increase in efficiency with prolongation of effort which he observed to the presence of relatively large static and postural components in the work. If this explanation of Simonson's results be true, then his experiments serve to confirm in man the teachings of studies of prolonged contraction in isolated muscles, but it would necessarily follow that any generalization from them as to the increased efficiency of muscular work in man with prolongation of effort must be limited to types of muscular work in which static and postural components predominate.

Summary.

Experiments have been made in order to ascertain whether the duration of effort affects the efficiency of muscular work in man when the load, rate of working and speed of shortening of the effector muscles are carefully controlled.

The type of work selected was pedalling a Martin's bicycle ergometer in which the muscular effort is primarily dynamic.

The total excess oxygen used for work and recovery (*a*) in short-spell efforts of 25 sec. to 1 min. duration, and (*b*) for 4 or 6 min. similar work, has been determined and the net efficiencies of performance calculated.

No material difference in efficiency was found whether the duration of work was 1 min. or less or 6 min., and it is concluded that provided the effort is primarily dynamic and the speed of muscular movement, load and rate of working are the same, the actual duration of work has little if any effect on the net efficiency of performance.

The results of this investigation are in accordance with predictions based on previous work, but they are in complete disagreement with the contentions of Simonson and Hebestreit who claim to have shown that a two to sixfold increase in efficiency occurs in man in muscular work of 6 to 10 min. duration as compared with similar effort exerted for 1 min. or less.

It is suggested that the predominant static and postural components in Simonson and Hebestreit's experiments accounted, at least in part, for their results, particularly in view of the known fact that the economy of maintaining a prolonged contraction is increased by previous activity.

I am greatly indebted to Prof. A. V. Hill for his helpful criticism and interest.

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ACTION OF ACETYLCHOLINE ON THE BRAIN AND ITS OCCURRENCE THEREIN.

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(*Received December* 6, 1933.)

The fact that the parasympathetic and the sympathetic nerves produce their effect by a specific chemical substance has been demonstrated by several workers. Dixon [1906], Howell [1906] and Sherrington [1925] suggested this possibility, while the work of Loewi [1921] on the amphibian heart definitely showed that parasympathetic stimulation liberated a substance resembling acetylcholine, and sympathetic stimulation liberated a substance resembling adrenaline. The work of Dale and Gasser [1926] on the denervated mammalian muscle and the subsequent demonstration of acetylcholine in the normal tissues by Dale and Dudley [1929] considerably helped in establishing the theory of humoral transmission. Literature concerning the theory of humoral transmission has been discussed by Dale in his Croonian lecture [1929], and the account given there shows that most of the work done has been concerned with the peripheral terminations of the nerves. Evidence is presented in this paper which suggests that humoral transmission may occur in the central nervous system.

Cushing [1931] found that injections of pituitrin and of pilocarpine into the brain ventricles produced similar effects, although these two drugs produced totally different effects when given intravenously. He suggested that the drugs when given intraventricularly stimulated centres situated in the wall of the ventricles. This work suggested the possibility of vago-mimetic drugs stimulating the vagal centres when injected intraventricularly, and the writer made experiments to compare the effects produced by central stimulation of the vagus and intraventricular injection of acetylcholine.

Vagal control of respiration has been a subject of considerable experimentation, and there is a certain degree of unanimity about the

effects produced by stimulation of the central end of the vagus on respiration. Rosenthal [1865] first showed that stimulation of the central end of the vagus caused inhibition of respiration, and many other workers have confirmed this observation since then. The effects produced depend on the strength of the stimulus; weak stimuli cause an inhibition and sometimes an acceleration of respiration, but a sufficiently strong current always produces inhibition.

Methods.

Cats were used in all experiments. The animals were anæsthetized with paraldehyde (1 c.c. per kg.) and ether or urethane (1·8 g. per kg.). Respirations were recorded by a tracheal cannula connected to a tambour and the blood-pressure by a cannula in the carotid artery. The vagi on both sides were dissected and cut in the middle of the neck. It was found that electrical stimulation of the central end of the cut vagus always caused a complete cessation of respiration if a sufficiently strong stimulus was used. Acetylcholine was injected into the ventricles through a small hole trephined at the junction of the coronal and sagittal sutures. Actions produced by the mechanical effects of the injection were controlled by injecting an equal volume of saline before and after the acetylcholine injections.

Effects of intraventricular injections of acetylcholine.

Fig. 1 shows the effect of an intraventricular injection of 0·5γ of acetylcholine. This produced a complete inhibition of respiration for about 30 sec. The drug given by this route produced no certain effect on the blood-pressure, for the slight but long continued fall in blood-pressure observed was probably due to the mechanical effects of cessation of respiration. The effects produced by intraventricular injections of acetylcholine in experiments on thirty cats are summarized in Table I. Table I shows that there is an extensive individual variation in the response to acetylcholine as regards the amount needed to produce an effect. The effects produced by acetylcholine were completely reversible, and several injections could be given to a single animal. In only two cases out of thirty-two did the animals fail to respond to a dose of 2γ of acetylcholine.

In a few experiments injections of less than 0·5γ acetylcholine produced acceleration of respiration, and in such cases it was found that stimulation of the central end of the vagus also produced augmentation of respiration.

A few experiments were made in which any secondary effects due to interference with ventilation were eliminated. Movements of the diaphragm of cats were recorded by a modification of the technique described by Thomas and Frank [1928].

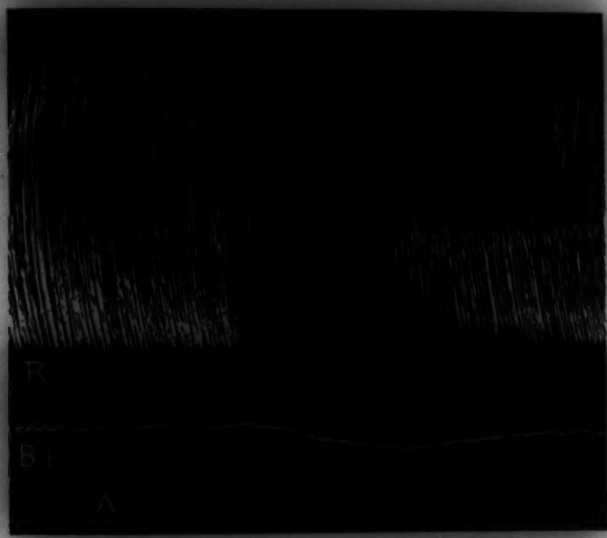

Fig. 1. Cat's respiration (R) and blood-pressure (B.P.). At *A* 0·5γ of acetylcholine injected in the right lateral ventricle. Time, 2 sec.

TABLE I. Effects produced by intraventricular injections of acetylcholine.

Dose of acetylcholine in γ	Incidence of effects: Cessation of respiration for 10 sec. or more	Depression and slowing of respiration	No effect on respiration
0·05–0·5	2	17	4
0·5 –1·0	4	1	2

Under artificial respiration a strip of diaphragm about 1 cm. wide and 4 cm. long was isolated in such a way that the phrenic nerve ran along the centre of the strip. The vessels accompanying the nerve were isolated and cut, but the nerve was left intact. The lower end of the strip was fastened to a fixed rod and the upper end connected to a writing lever. The strip,

if moistened with Locke's solution, contracted regularly for a considerable time.

Fig. 2 shows a typical record of an experiment. This figure shows that

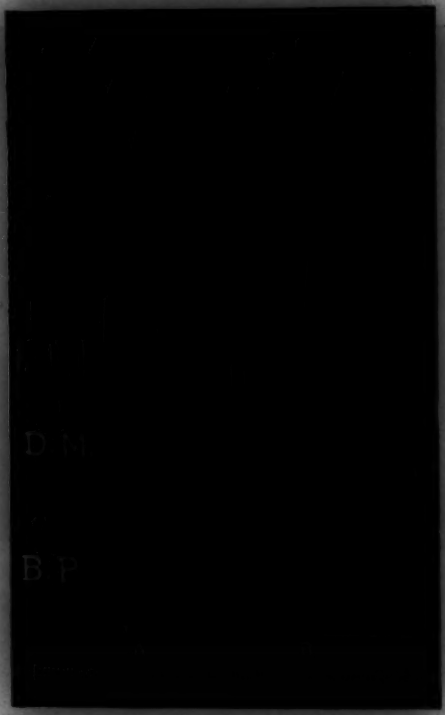

Fig. 2. Cat's diaphragm strip movements (D.M.) and blood-pressure (B.P.). At *A*, Stimulation of the central end of the left vagus. *B*, Injection of 0·1 γ of acetylcholine in the third ventricle. Time, 2 sec.

the action of intraventricular acetylcholine on respiratory movements occurs when artificial respiration is maintained and when the muscle is isolated from circulation. Therefore the drug must act on the central nervous system.

Small doses of atropine (0·5 mg. per kg.) suffice to abolish the effects produced by intravenous injections of acetylcholine and the latter are augmented by small doses of physostigmine (0·1 mg. per kg.). The inhibition of respiration produced by intraventricular injections of acetylcholine is not affected by atropine when the latter drug is given either intravenously or intraventricularly. Furthermore, atropine does not affect the inhibition of respiration produced by stimulation of the central end of the vagus.

Atropine was given in doses of 0·1–1 mg. intravenously (seven experiments) and 0·02–0·1 mg. intraventricularly (three experiments). In no case was any change observed in the effects produced by intraventricular injections of acetylcholine (0·1–0·5γ), given before and after the atropine.

The effects of stimulation of the central end of the vagus were observed in thirty experiments before and after similar doses of atropine, and in no case did this drug cause any change in the effects produced.

Engelhart and Loewi [1930] showed that physostigmine inhibited the hydrolysis of acetylcholine by an esterase commonly present in the blood. If therefore the inhibiting action on respiration seen after stimulation of the central end of the vagus is due to liberation of acetylcholine, it might be expected to be enhanced by intravenous and intraventricular injections of physostigmine. It was however found that neither intravenous nor intraventricular injections of physostigmine materially altered the effects of vagus stimulation or produced any other detectable change in the effects of intraventricular acetylcholine.

Physostigmine was given in doses of 0·1–2 mg. intravenously to ten animals, and intraventricularly to fourteen animals. In no case was any change observed in the effects produced before and after these injections by either central stimulation of the vagus or by intraventricular injections of acetylcholine.

These experiments show that the respiratory inhibition produced by intraventricular administration of acetylcholine is neither paralysed by atropine nor augmented by physostigmine, and that the same is true of the effects produced by stimulation of the central end of the vagus.

There are, however, various peripheral actions of the vagus and of acetylcholine which are not antagonized by atropine. For example, atropine does not abolish the augmentor effect of the vagus on the intestinal movements. Similarly the vaso-dilator effect produced by antidromic stimulation of the sensory nerves is not affected by atropine, although this abolishes the vaso-dilator action of acetylcholine. The anomalies observed in regard to the antagonism of acetylcholine by

atropine have been discussed by Dale [1929]. Dale and Gaddum [1930] observed that the action of acetylcholine on denervated mammalian muscle is not abolished by atropine. Dale [1929] in his general discussion pointed out that, in various other cases, effects produced by nerve stimulation which mimicked acetylcholine action showed a similar difference as regards the effect of atropine. He concluded that the failure of atropine to paralyse the vaso-dilator effect of antidromic stimulation of sensory nerves did not disprove the hypothesis that this effect was due to liberation of acetylcholine.

The experiments described above show that intraventricular acetylcholine and central stimulation of the vagus produce very similar inhibition of respiration. The simplest explanation is to assume that central stimulation of the vagus liberates acetylcholine, and this theory is not disproved by the failure of atropine and physostigmine to influence the response, because these drugs do not influence the response of intraventricular administration of acetylcholine.

Effects of stimulation of the central end of the vagus depend upon the strength of the stimulus. With a weak current it is not unusual to find that, during the period of stimulation, there is actually an increase in the rate as well as depth of respiration and the action of inhibition comes on after cessation of the stimulus. In animals with normal respirations the apnœa might conceivably be a physiological one following hyperpnœa. If, however, this effect is studied in animals under artificial respiration whose respiratory movements are studied by the diaphragm strip method, it becomes obvious that the apnœa is a true effect of vagal stimulation. It is easier to explain these delayed effects by a secretory theory than by any other, for it can be argued that during the period of stimulation a chemical substance is liberated which persists after cessation of the stimulus. The increase in the rate and depth of respiration may be ascribed to the immediate effect of stimulation of an afferent nerve, while the delay in the cessation of respiration which occurs after cessation of the stimulus is an effect that suggests the liberation of an active chemical substance.

Beattie, Brow and Long [1930] observed that stimulation of the central end of the vagus in cats under light chloroform anæsthesia precipitated irregularities in hearts which were beating regularly before the nerve stimulation. In a certain proportion of cases I have observed irregularities of the heart precipitated after stimulation of the central end of the vagus. Injections of small doses of acetylcholine (0·1–0·5γ) in the lateral ventricle of such animals also produced irregularities of the heart.

It would appear from the experimental data given above that there is some reason to believe that an acetylcholine-like substance might be liberated in the brain during stimulation of the central end of the vagus. If it is liberated in the brain it should eventually appear in the cerebro-spinal fluid. Attempts were therefore made to see if any such substance could be detected in the cerebro-spinal fluid after stimulation of the central end of the vagus.

Occurrence of acetylcholine in the cerebro-spinal fluid and the brain.

Cats were used for these experiments, artificial respiration was employed and the central end of the vagus was stimulated with a current sufficiently strong to produce a cessation of respiration. Immediately after or during the nerve stimulation, the cisterna magna was tapped by a medium-sized needle and about 1 c.c. of clear cerebro-spinal fluid withdrawn. Another sample was collected about 20 min. later, and the two samples were tested on the blood-pressure of cats and on isolated ventricular strips of the frog's heart, to determine whether any acetylcholine-like substance was present. The object of these experiments was to determine whether a greater amount of depressor substance was present in the cerebro-spinal fluid after stimulation of the central end of the vagus than was present in the control specimen. The simple comparative nature of this estimation makes it unnecessary to discuss the nature of the effects produced by the injections.

Injections of normal cerebro-spinal fluid intravenously produce very varying results on the blood-pressure of cats, and the exact significance of the results obtained from such experiments is therefore obscure. In these experiments only the comparative action was taken into consideration to see if any depressor substance was liberated in the cerebro-spinal fluid after central vagal stimulation when no such substance could be detected in the normal cerebro-spinal fluid, or if any depressor substance was present in the normal whether its quantity was enhanced after vagal stimulation.

Thirty experiments were made, and in twenty-seven no certain difference was observed as regards the vaso-depressor actions produced by the intravenous injections of the two samples of cerebro-spinal fluid. In two cases the sample collected after vagal stimulation produced the greater action, but in one case the control sample showed the greater action.

The cerebro-spinal fluid also was tested for acetylcholine upon the frog's heart. The fluid was diluted with half its volume of water and was applied to isolated ventricular strips according to the method used by Clark [1926]. Twenty-one experiments were made and the results were negative in nineteen cases, but in two cases the cerebro-spinal fluid obtained after vagal stimulation produced a depressor action, which was abolished by atropine, although the control sample produced no effects.

The tests on cats' blood-pressure and on the frog's heart should detect the presence of about 0·01γ of acetylcholine in 1 c.c. (1 in 100,000,000). The general result of the tests is to show that in the great majority of cases the acetylcholine content of the cerebro-spinal fluid, both before and after vagal stimulation, is less than 1 in 100,000,000, but that occasionally vagal stimulation causes the appearance in the cerebro-spinal fluid of an acetylcholine-like substance with an action equivalent to a concentration of acetylcholine of about 1 in 100,000,000.

Attempts also were made to demonstrate the presence of acetylcholine in the brain.

Since the observation of Schäfer and Moore [1896] that intravenous injections of brain extracts produce a fall in blood-pressure, there has always been a controversy about the nature of the substance in those extracts. Mott and Halliburton [1899] thought it to be choline, while Vincent and Sheen [1903] did not think it to be so. The controversy narrowed to a smaller issue after some time, the point of discussion being whether the "important" substance present was choline or not. Apart from pharmacological tests acetylcholine was demonstrated in the brain extracts by chemical methods also [Gulewitsch, 1899]. Later researches [Miller and Miller, 1911; Major and Weber, 1929, 1930; Major, Nanninga and Weber, 1932; Euler and Gaddum, 1931] have shown that there are several substances present in the brain which have a depressant action on the blood-pressure. Chang and Gaddum [1933] estimated the acetylcholine equivalent of dog's brain as 0·4γ per g. of tissue. This value is lower than those found for many other tissues of the dog such as the intestines and the auricle.

The author [1933] estimated the acetylcholine content of the brain by the following method.

Fresh brains of cats and rabbits were used for this purpose. The extracts were prepared by macerating different parts of the brain in an acetone-water-acid mixture containing 75 p.c. acetone, 15 p.c. distilled water and 10 p.c. N/100 acetic acid. The emulsion was allowed to stand for 24 hours at 5° C., filtered and evaporated to dryness *in vacuo* at room temperature.

The residue was dissolved in distilled water and used for injections as crude extract.

The pharmacological properties of this extract were found similar to those of acetylcholine. It lowered the blood-pressure after an intravenous injection, and the nature of the fall so produced was indistinguishable from that produced by acetylcholine. It produced contraction of the isolated gut of the rabbit, depressed the perfused frog's heart and inhibited the respiration of the cat after an intraventricular injection, in the same manner as acetylcholine.

The acetylcholine equivalent of the extracts was estimated by measuring the fall in cat's blood-pressure produced by intravenous injections. Results obtained are summarized in Table II. The value obtained in the case of the cat agrees with that obtained by Chang and Gaddum [1933] in the dog.

TABLE II.

		Acetylcholine equivalent of extracts of the basal ganglia in γ per g. fresh tissue	
Animal	No. of expts.	Range of figures	Mean
Cat	10	0·2 –1·3	0·47
Rabbit	4	0·04–1·0	0·15

Extracts were also made of the cerebral cortex and cerebellum. The cortical extracts were only slightly weaker than the basal ganglia extracts, but the cerebellar extracts showed only half this activity.

The nature of the depressor substances present in the brain has been discussed by Euler and Gaddum [1931] and the general principles regarding biological demonstration of acetylcholine by Chang and Gaddum [1933]. These workers have shown the multiplicity of biologically active substances present in tissue extracts. Their work shows that the following tests for acetylcholine deserve special consideration: (1) production of a vaso-depressor action which is abolished by atropine, and (2) production of a contraction of the isolated rectus abdominis of the frog which is augmented by physostigmine.

Dr Stedman kindly provided me with a purified acetylcholine esterase isolated from horse blood [1932]. This esterase breaks down acetylcholine, its action is rapid for it completely destroys acetylcholine in high dilutions in less than 5 min., and as far as is known its action is specific for choline esters. This substance therefore provides a simple method for testing the presence of acetylcholine in extracts. By itself it has no detectable pharmacological action on blood-pressure or respira-

tion, and produces no alteration in the records of these so as to interfere with quantitative estimations. Another advantage of this method is that injections of extracts and acetylcholine can be repeated in the same animal and results obtained thus properly controlled. I have used the following technique to demonstrate acetylcholine in the brain extracts.

The extract to be tested is divided in three equal portions. The first is injected intravenously and the effects on the blood-pressure noted. The esterase is added to the second and, after about 5 min., this is injected intravenously as before. The third is used as a control to see if the response is the same as the first one. Usually the first and third injections show the same degree of vaso-depression while the second shows either no fall of blood-pressure at all or a comparatively smaller fall.

Fig. 3. Cat's blood-pressure (B.P.). *A*, 0·5 c.c. of basal ganglia extract given intravenously *B*, 0·5 c.c. of the same extract treated with esterase before administration. Time, 10 sec.

Brain extracts tested in this way show that the relative quantities of acetylcholine and the other depressor substances present in the brain vary a good deal. It is not unusual to find an extract whose depressor activity is completely abolished by treating it with the esterase. Fig. 3 shows the results obtained in one such experiment. The first injection produced a fall of blood-pressure which was completely abolished by the action of the esterase.

I have obtained about the same results with atropine. Atropine abolishes the depressor activity of some extracts only partially and of others completely. Fig. 4 shows the complete antagonistic action of atropine on the depressor effect of the basal ganglia extract.

The extracts were also tested on the isolated rectus abdominis of frogs (*R. temporaria* and *esculenta*). Application of extracts to these produced

a contraction which was markedly enhanced by soaking the muscle in a 1 : 100,000 solution of physostigmine. This effect is a specific test for acetylcholine, and it can be concluded from all these experiments that one of the depressor substances present in the brain extracts is acetylcholine.

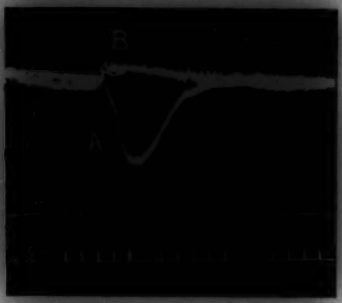

Fig. 4. Cat's blood-pressure. Drum turned back to get superimposed tracings. 0·5 c.c. of basal ganglia extract injected twice at the signal. *A*, Tracing before intravenous atropine. *B*, Tracing after atropine. Time, 10 sec.

Discussion.

The presence of an acetylcholine-like substance in the brain, its higher concentration in the basal ganglia which is supposed to be the seat of a number of visceral activities and its occasional occurrence in the C.S.F. after vagal stimulation appear to be of some significance when viewed along with the fact that stimulation of the central end of the vagus, and injections of small quantities of acetylcholine in the ventricles of the brain have a similar action on respiration. The delayed effects of vagus stimulation and the occasional production of cardiac irregularities after vagal stimulation and intraventricular acetylcholine injections also support the view of humoral transmission in the central nervous system. The evidence so far collected in support of the theory of humoral transmission is chiefly indirect, the only direct evidence being the occasional occurrence of a vaso-depressor substance in the C.S.F. after vagal stimulation when no such substance could be detected in the normal C.S.F. It has however been possible to present certain data which suggest that such a transmission is possible.

Summary.

1. Injection of acetylcholine (0·1–1·0γ) into the cerebral ventricle of cats causes effects similar to those produced by stimulation of the central end of the vagus, namely arrest of respiration and, occasionally, cardiac irregularity.

2. Intraventricular injections of acetylcholine (0·1–1·0γ) do not produce a fall of blood-pressure.

3. The effects produced by intraventricular injections of acetylcholine are not affected by administration of large doses of atropine or of physostigmine.

4. Extracts of the basal ganglia contain a depressor substance which shows the properties of acetylcholine in a variety of biological tests. The acetylcholine equivalent of this substance is 0·4γ per g. of basal ganglia in cats.

5. The cerebellum and cortex contain less of this substance than the basal ganglia.

I desire to acknowledge my indebtedness to Prof. A. J. Clark for suggestions and constant help throughout the course of these experiments. The expenses of the research were defrayed by a grant from the Moray Fund of Edinburgh University.

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ESTIMATION OF FIBRINOGEN AND THROMBIN.

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(*Received November* 3, 1933.)

The objective of the experiments about to be described is the estimation of fibrinogen and thrombin. The former is estimated in terms of the amount commonly present in human blood plasma to which 0·3–0·7 p.c. of trisodium citrate has been added: this amount will be shown later to be remarkably constant. The source of thrombin is the venom of *Echis carinatus*, a small Indian viper; the unit of concentration of thrombin is taken as that concentration which will completely convert into fibrin the fibrinogen contained in normal human plasma.

The methods employed for estimation are based upon the effect on fibril formation of diminution of one or other component of the coagulant mixture [Barratt, 1915]. The term "coagulation time" is used in this investigation to signify the time of onset of coagulation indicated by the first appearance, under dark-ground illumination, of fibrils of fibrin. For dilution of plasma or venom 0·85 p.c. NaCl solution was employed. The temperature of experiment was 16–18° C.

In the observations given in Tables II and III equal parts of citrated plasma and venom solution were taken, in all other observations the proportion was nine of the former to one of the latter. The actual concentration of plasma and venom present in the mixed liquid in each observation is given in the tables in the second and third columns respectively.

Estimation of fibrinogen.

If equal amounts of thrombin are added to a series of increasing dilutions of fibrinogen, contained in citrated human plasma, the coagulation time is found to increase with each successive dilution, as is illustrated by the experiment set forth in Table I and by the curve to the

TABLE I. The effect of dilution of fibrinogen upon coagulation time (cp. Table IV).

Observation	Concentrations used for coagulation: Plasma (z) p.c.	Concentrations used for coagulation: Venom (y)	Coagulation time (t) in min.: Observed	Coagulation time (t) in min.: Calculated for $t=5{\cdot}15/z^{0{\cdot}5}$
1	88	1 in 640,000	5·00	5·5
2	44	" "	9·00	7·8
3	22	" "	11·00	11·0
4	11	" "	14·75	15·6

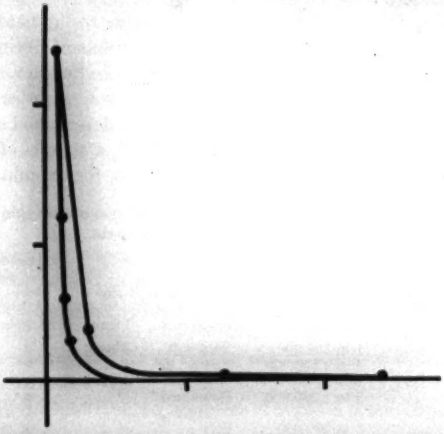

Fig. 1. The effect upon coagulation time of dilution of fibrinogen is shown in the curve to the left (Table I) and of thrombin to the right (Table IV). Scale: abscissæ, periods of 100 min.; ordinates, concentration of plasma, 39 p.c. (curve to left), or venom, 1 in 1,540,000 (curve to right). Obs. 1–7, Tables I and IV, form a single series.

left in Fig. 1. In this and other experiments the relation between coagulation time (t) and relative concentration of fibrinogen, *i.e.* percentage of plasma (z), is given by the equation

$$t = \frac{a}{z^p}, \qquad \text{.....(1)}$$

where a is a constant varying in individual experiments and having the value 5·15 for that given in Table I. The constant p lies within a limited range of values: its average value in numerous experiments was 0·5.

Relative values of the content of fibrinogen in different solutions of fibrinogen may be readily obtained by equation (1) from the coagulation

times observed when the same amount of thrombin has been added to each solution. If the concentration of fibrinogen is very low some difficulty may, however, be experienced in ascertaining the exact moment at which fibrils first appear, the thickness of the fibrils becoming exceedingly small so as to render rapid recognition impossible. In a dilution containing 0·75 p.c. of normal human plasma fibrils cease to appear and "setting" is difficult to detect or no longer recognizable (Table II). The value of p, it may be noted, was found to range in different experiments between 0·35 and 0·60.

Another method of estimation which avoids the difficulty of exact determination of coagulation time in high dilutions of fibrinogen, when fibrils are of liminal visibility, is the following, based upon ascertainment of the point at which visible fibril formation ceases. A series of dilutions of the liquid whose content of fibrinogen is to be determined is made, and to each the same amount of thrombin is added, a sample of each being then placed in a glass cell, sealed with vaseline, for examination under

TABLE II. The dilution of plasma at which addition of thrombin fails to give rise to visible fibrils of fibrin.

	Concentrations used for coagulation		Condition of clot		Description of plasma	
Observation	Plasma p.c.	Venom	Consistence	Fibrils	Sample	Age (days)
1	0·75	1 in 320,000	Very soft	Absent	*a*	0
2	,,	1 in 1,280,000	Soft	,,	*b*	0
3	,,	1 in 320,000	Very soft	,,	*c*	1
4	,,	1 in 1,280,000	Remained liquid	,,	*d*	1
5	,,	1 in 320,000	Soft	,,	*a*	7
6	,,	1 in 5,120,000	Very soft	,,	*b*	43

dark-ground illumination at the end of 24 hours. The dilution at which visible fibrils cease to be formed is in this way ascertained. By a comparison of determinations thus made for different solutions, an estimate of the corresponding relative concentrations of fibrinogen is obtained. It will be noted that, in this method, only the presence or absence of fibrils is noted, no attempt being made to determine the time at which fibrils become visible. The dilution at which visible fibril formation ceases corresponds, as already mentioned, to a concentration of normal human plasma of 0·75 p.c.

By the use of these two methods relative estimations of fibrinogen may be made. In order to obtain absolute determinations a standard value of fibrinogen is required. This cannot be given in terms of a definite amount of fibrinogen owing to the circumstance that it is not at present

possible to obtain fibrinogen in a state of purity or even in a stable form, but a natural unit of concentration is furnished by human blood plasma, which in health appears to exhibit very small variation of its content of fibrinogen. The limited range of variation is shown by the appearance of nearly identical coagulation times when simultaneous determinations are

TABLE III. Experiment illustrating the extent of variation of content of fibrinogen in human plasma.

Observation	Concentrations used for coagulation: Plasma p.c.	Concentrations used for coagulation: Venom	Coagulation time (min.)	Source of plasma
1	47	1 in 1,767,000	15·0	*A*
2	„	„ „	15·0	*B*
3	„	1 in 2,580,000	19·5	*A*
4	„	„ „	19·5	*B*
5	„	1 in 394,000	5·3	*A* (1)
6	„	„ „	5·3	*A* (14)
7	„	„ „	5·2	*A* (66)
8	„	„ „	5·3	*A* (98)
9	„	„ „	5·5	*A* (182)
10	„	„ „	5·2	*A* (198)

made with citrated plasma from different individuals, equal amounts of thrombin being added in each case. The same is indicated still more strikingly when comparative tests are made with citrated plasma obtained at different times from the same individual, such determinations being rendered possible by the use of venom, deterioration of which at 0° C. is very slow [Barratt, 1932]. Illustrative observations are shown in Table III. In the first four, two samples of citrated plasma obtained from different individuals are found to have identical coagulation times when tested shortly after collection (1 and 2) and again 2 days later (3 and 4). The next six observations (5–10) are made with samples of human plasma from the same subject, taken at intervals (given by the figures in brackets in column 5) during a period of 198 days. Since the coagulation time is practically identical in these experiments, it follows that neither the content of thrombin in the solution of venom employed, which was made up two years before the first experiment, nor the content of fibrinogen in the samples of plasma, varied in any marked degree during the period of experiment. The plasma in Obs. 5–10, it may be observed, was examined shortly after collection of blood except in Obs. 10, in which it was 24 hours old when used. The content of fibrinogen in normal human plasma thus furnishes a standard, which may be taken to represent unit concentration. Estimations in terms of this unit may be made in liquids containing fibrinogen by either of the two methods given above.

Estimation of thrombin.

If thrombin is added to plasma in diminishing amounts, coagulation takes place with increasing slowness. The relation between the time required for "setting" to occur (t) and the amount of thrombin added (y) was found by Fuld [1902] to be given by the equation

$$\log \frac{t_1}{t_2} = 0{\cdot}585 \log \frac{y_2}{y_1}. \qquad \ldots\ldots(2)$$

Martin [1905], using snake venom, observed the relation

$$ty = \text{constant} \qquad \ldots\ldots(3)$$

when the quantity of thrombin added was small: with large amounts of thrombin the observed values of t were greater than the calculated periods. Rettger [1909] also noted that equation (3) failed if a maximum amount of thrombin was employed, further increase of thrombin no longer causing a diminution of the time of coagulation. Similar results were obtained by Barratt [1915].

If, however, the plasma employed is citrated and the coagulation period taken as the time required for the formation of visible fibrils of fibrin (this being capable of more exact measurement than the occurrence of "setting") the relation

$$t = \frac{b}{y^q} \qquad \ldots\ldots(4)$$

is obtained, where b is constant only for individual experiments, while q varies within narrow limits, ranging in different experiments between 0·65 and 0·75. This is illustrated by the experiment detailed in Table IV

TABLE IV. The effect of dilution of thrombin upon coagulation time (cp. Table I).

Observation	Concentrations used for coagulation: Plasma (z) p.c.	Concentrations used for coagulation: Venom (y)	Coagulation time (t) in min.: Observed	Coagulation time (t) in min.: Calculated for $q=0{\cdot}000475/y^{0{\cdot}7}$
1	88	1 in 640,000	5	5·5
5	,,	1 in 5,760,000	28	25·5
6	,,	1 in 51,840,000	128	119·0
7	,,	1 in 155,520,000	246	257·0

and shown in the curve to the right in Fig. 1. In this experiment the constant b has the value 0·000475, and the exponent q is taken at 0·7, the coagulation times calculated in the fifth column agreeing, it will be seen, fairly closely with the observed periods. Experiments of this type afford a valuable method of making comparative estimations of thrombin in venom dilutions.

Absolute estimations may also be made. These are based upon the relation observed between the amounts of fibrinogen and thrombin taking part in the production of fibrin. If thrombin is added to citrated plasma it is usually found that the fluid expressed from the resulting clot at the end of several days, when the interaction between fibrinogen and thrombin may be regarded as complete, contains thrombin if a relatively large amount of thrombin has been added, while, on the other hand, fibrinogen is present if only a relatively small amount of thrombin is used. This is shown by the addition of citrated plasma to the expressed fluid in the first case, and of venom in the second, when clotting occurs. If, however, fibrinogen and thrombin are mixed in suitable proportions, the expressed fluid is found to be free from either, that is to say, the further addition of plasma or thrombin no longer causes clotting. This is illustrated in Table V: in Obs. 1, 2 and 3 fibrinogen is absent from the expressed fluid,

TABLE V. Determination of the amount of thrombin required to convert fibrinogen into fibrin.

Observation	Concentrations used for coagulation: Plasma p.c.	Concentrations used for coagulation: Venom	Approximate ratio of fibrinogen to thrombin	Content of fluid expressed from clot: Fibrinogen	Content of fluid expressed from clot: Thrombin
1	47	1 in 21,500,000	50,000 : 1	–	+
2	70	1 in 41,000,000	143,000 : 1	–	+
3	80	1 in 71,000,000	284,000 : 1	–	+
4	80	1 in 144,000,000	576,000 : 1	+	–
5	10	1 in 144,000,000	72,000 : 1	–	+
6	86	1 in 331,000,000	143,000 : 1	–	+
7	10	1 in 568,000,000	284,000 : 1	–	+
8	10	1 in 1,135,000,000	576,000 : 1	+	–

while in Obs. 4 it is present, the converse being true of thrombin. If it is assumed that fibrinogen and thrombin are in corresponding amounts midway between Obs. 3 and 4, the relation between fibrinogen and thrombin would be in the neighbourhood of 384,000 : 1, the plasma employed being assumed to contain 0·5 p.c. of fibrinogen and venom being regarded as pure thrombin. This proportionality, which does not seem to be altered by dilution—in Obs. 5–8, in which the concentrations used are about one-eighth of those in Obs. 1–4, a similar relation is indicated—renders possible the estimation of thrombin in terms of a unit, for it is obvious that if normal human plasma is taken as representing unit concentration of fibrinogen, then the concentration of thrombin just sufficient to convert all the fibrinogen present in the plasma into fibrin must be taken to represent unit concentration of thrombin. It is, however, preferable to indicate this concentration by the corresponding coagulation time rather

than to attempt to express it in terms of thrombin. Experiments made to determine this period—that is to say, the coagulation time when thrombin is added in amount just sufficient to convert all the fibrinogen present in undiluted normal human plasma into fibrin—were found to give a mean value of approximately 135 min., corresponding to a 1 in 77,500,000 concentration of the sample of venom used in Obs. 1–7, Tables I and IV.

Using this value of t for unit concentration, the following illustration of the determination of titre of a solution of thrombin may be given. In Obs. 1, Table I, the observed value of t for 88 p.c. plasma is 5 min. The value of t for undiluted plasma is by equation 1, p. 423,

$$5 \times \left(\frac{0{\cdot}88}{1{\cdot}00}\right)^{0{\cdot}4} = 4{\cdot}7 \text{ min.}$$

The concentration of thrombin employed in Table I is, therefore, by equation 4, p. 426, $\left(\frac{135}{4{\cdot}7}\right)^{1{\cdot}48} = 121$ times unit concentration.

Summary.

1. Methods of estimation of fibrinogen and thrombin, based upon the observed effect of dilution, are described.

2. As units the following are employed:

(*a*) Unit concentration of fibrinogen: the concentration present in normal human plasma.

(*b*) Unit concentration of thrombin: the concentration which is just sufficient to convert all the fibrinogen present in normal human plasma into fibrin.

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THE VASO-DILATOR ACTION OF ADRENALINE.

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It is now generally recognized that very small doses of adrenaline given intravenously, especially in certain conditions of anæsthesia [Macdonald and Schlapp, 1926; Vincent and Curtis, 1927], can produce a fall in blood-pressure in the cat or in the dog. It has also been shown that these minute amounts of adrenaline cause vaso-dilatation which apparently has its site in the capillary field [Dale and Richards, 1918]. Earlier work by Cannon and Lyman [1913] indicated that this peripheral dilatation was not limited to any particular region or tissue of the body, for they were able to obtain a depressor effect with adrenaline after clipping carotid, subclavian and iliac arteries, and the fall in pressure was not much less than that seen when these arteries were open and the superior and inferior mesenteric, cœliac axis and renal arteries were clamped. Experiments by Gruber [1929], however, on unanæsthetized animals showed that small doses of adrenaline regularly caused dilatation in the vessels of voluntary muscle, judged by the increased venous outflow, but that not in every case did the general blood-pressure fall; this the author interpreted as indicating that adrenaline dilated muscle vessels but, in those cases where the pressure remained constant, constricted vessels in other regions of the body. There is much evidence to show that the action of adrenaline varies both qualitatively and quantitatively in different parts of the body; thus, the coronary vessels are dilated [Hammouda and Kinosita, 1926], the vessels of voluntary muscle are also dilated [Hoskins, Gunning and Berry, 1916; Hartman and McPhedran, 1917; Gruber, 1929; Clark, 1930] except where large doses are given [Erlanger and Gasser, 1919; Gunning, 1917; Gruber, 1929], while skin vessels appear to be uniformly constricted [Hoskins, Gunning and Berry, 1916; Clark, 1930]; the splanchnic vessels also respond by constriction, but, if the adrenaline given raises

the general blood-pressure, constriction is changed to dilatation which is partly reflex and partly passive [Clark, 1930]. The vaso-dilatation caused by adrenaline in skeletal muscle is a purely peripheral effect, for it has been seen after cutting all nerves to a limb [Dale and Richards, 1918] and also in the isolated perfused limb [Dale and Richards, 1927], but there is no conclusive evidence to show whether or not adrenaline in small doses can exert on all minute vessels in the body a dilator action which is definitely peripheral.

It seemed that a reinvestigation of the problem was necessary to determine whether or not vessels in the skin and in the splanchnic area would respond as do those in skeletal muscle, and, if possible, to determine under what conditions dilatation or constriction predominates.

Cats under chloralose anæsthesia were used and vascular changes were determined by recording the venous outflow from muscle, skin or intestine, clotting being prevented by intravenous injection of "Novirudin." After falling on an electric drop recorder, the blood was collected in a beaker kept at 39° C. by means of a water jacket and was returned to the animal by a burette connected with one external jugular vein. In the case of muscle a cannula was inserted into the femoral vein and, to ensure that the flow came only from muscle, the leg was tightly ligated immediately above the ankle and all cutaneous veins tied; in some cases the leg was skinned and the skin replaced with the help of sutures so that all chance of anastomosis between skin and muscle was prevented.

Blood flow from the skin was observed by recording the outflow from the superficial femoral vein; special care was taken to keep the vein in the vicinity of the cannula moist and covered by skin to prevent contraction.

Intra-arterial injection of adrenaline was made by inserting the needle of a suitable syringe into the stump of the common origin of the internal iliac arteries, after tying off all branches; the needle could be pushed up till the point was in the stream of the external iliac. Repeated injections were given by leaving the syringe *in situ*, taking precautions to prevent blood entering and pushing up the piston.

In the case of the mesenteric vessels a loop of intestine was isolated from the rest of the gut and a cannula inserted into the vein draining the loop. Intra-arterial injections were given through the stump of a branch of the artery supplying the loop. It sometimes happened that the blood flow through the intestine was irregular due to contractions of the intestinal wall, but this could be prevented by intravenous injection of atropine which appeared in no way to interfere with the action of adrenaline to be described below.

Special care was taken to maintain the temperature of the cat at 39·2° C. which is the mean normal found by Lee and Scott [1914], because Rein [1930] is of the opinion that adrenaline dilates muscle vessels only when the body temperature is subnormal or when the muscle is active. A few experiments were made at temperatures above and below the normal.

Vessels of voluntary muscle. The sequence of events following the intra-arterial injection of a minute dose of adrenaline is shown in Fig. 1.

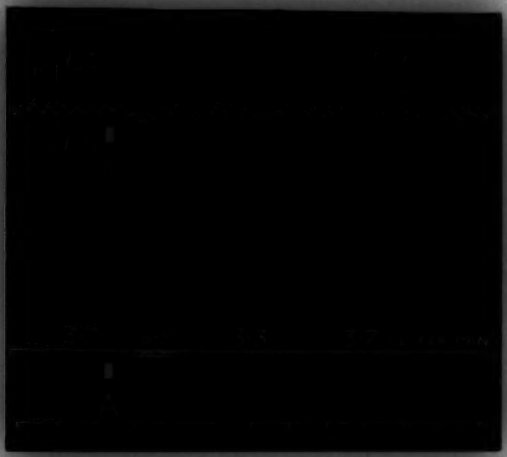

Fig. 1. Blood-pressure and venous outflow from muscles of hind leg. Skin of leg separated from underlying tissues and held in place by stitches. Temp. 39·2° C. Time in 10 sec. At A, 0·05 c.c. adrenaline 1×10^{-4} into iliac artery.

There is a slightly shorter latent period in the blood-pressure record than in that of the blood flow from the muscle, which is to be expected because of the time which must elapse before the increased flow reaches the cannula; following the latent period and during the slight fall in blood-pressure the venous flow is increased for a time and then decreased before it returns to its original rate. These changes are almost invariably seen after giving adrenaline as described; they appear to be independent of any temperature changes likely to be found under physiological conditions, for Fig. 2 was obtained from a cat whose rectal temperature had

been allowed to fall to 36·0° C., *i.e* 3·2° C. below the mean normal temperature, while a similar response was seen at 41·3° C.

Adrenaline dilatation has also been seen in limbs denervated from 5 min. to 2 hours before experiments where no sign of muscular activity was evident. These results, therefore, do not confirm those of Rein [1930] already referred to.

An effect, similar to that shown in Fig. 1, has been obtained by injecting intra-arterially 0·1 c.c. of blood from the suprarenal vein of a second cat under chloralose anæsthesia during stimulation of the splanch-

Fig. 2. Blood-pressure and muscle blood flow. At † 0·05 c.c. adrenaline 1×10^{-4} into iliac artery. Temp. 36·0° C. Time in ½ min.

nic nerve; injection of the same amount of blood from the inferior vena cava before splanchnic stimulation gave no dilatation.

By giving repeated doses of adrenaline at short intervals the constrictor effect of one injection can be eliminated or cut short by a subsequent injection (Fig. 3). Where the time of injection is prolonged as in Fig. 4 B, it will be seen that no definite constriction occurs during the injection; in fact, the rate of flow is actually increased, and this cannot be attributed to the volume of fluid injected, as 0·1 c.c. is equivalent to less than two drops of the size here recorded. In Fig. 3 the same amount of adrenaline was given, not continuously as in Fig. 4 B, but with a pause

between the five fractions into which the dose was divided. The dilator effects of the first two fractions are combined and the third appears to

Fig. 3. Blood-pressure and muscle blood flow. At each white area 0·03 c.c. adrenaline 1×10^{-6} into iliac artery. Figures above the drop record indicate flow in c.c. per min.

Fig. 4. Blood-pressure and muscle blood flow. A. 0·10 c.c. adrenaline 1×10^{-6} at white area. B. 0·15 c.c. adrenaline 1×10^{-6} at enclosed area. Time in 10 sec. Figures above the drop record indicate flow in c.c. per min.

have less effect than any of the others although it definitely cuts short the constrictor phase judged by the duration of this latter after the third, fourth and fifth fractions. The dilatation following the fourth fraction is

slightly less than that following the fifth, and these differences may be related to the progressively longer intervals between the beginning of any two injections.

The suggestion of Cannon and Lyman [1913] that the production of dilatation or constriction by adrenaline depends on whether the vessels are initially constricted or dilated is not borne out by experiments in which repeated small amounts of adrenaline are given so that the dilator phase is prolonged; the first two injections in Fig. 3 give an example of this. Nor is their point of view upheld by the fact that dilatation may be seen when the blood pressure is definitely below the "critical level" given by them (Fig. 5).

Fig. 5. Blood-pressure and muscle blood flow. At † 0·05 c.c. adrenaline 1×10^{-4} into iliac artery.

Although the phenomena described above are those usually seen as a result of giving small doses of adrenaline into an artery, exceptions have been observed in which only a constriction and no dilator phase occurs with the smallest amount of adrenaline which gives a response. Other exceptions, still rarer, are those where only constriction is seen with a very small dose, but a brief dilatation occurs in the response to a larger dose. These apparently anomalous cases require further investigation.

Intestinal vessels. The effect of small intra-arterial injections of adrenaline on the blood flow through the intestinal vessels is shown in Fig. 6. The smallest dose which has any detectable effect always causes vaso-constriction, judged by the diminished venous outflow. It might be objected that the result here shown would be more likely to occur in gut,

the vessels of which had lost their tone due to the necessary operative manipulations, but in all experiments the intestine was handled as little

Fig. 6. Blood-pressure and venous flow from loop of intestine. At A, 0·05 c.c. adrenaline 1×10^{-6} intra-arterially. At B, 0·01 c.c. adrenaline 1×10^{-6} intra-arterially. At C, 0·02 c.c. adrenaline 1×10^{-6} intra-arterially. Time in 10 sec. The figures above the drop record indicate flow in c.c. per min.

Fig. 7. Blood-pressure and venous flow from loop of intestine. Between A and B, 0·6 mg. ergotoxine per kg. intravenously. A. 0·01 c.c. adrenaline 1×10^{-6} intra-arterially. B. 0·10 c.c. adrenaline 1×10^{-6} intra-arterially. Time in 10 secs. The figures above the drop record indicate flow in c.c. per min.

as possible and was always left in the abdominal cavity covered by omentum. The blood-pressure does not suggest any degree of shock and, moreover, the same response to adrenaline shown in Fig. 6 can be obtained

during the rise in blood-pressure caused by an intravenous injection of posterior pituitary extract. It seems therefore that the minute vessels of the intestine respond to the direct action of adrenaline in a manner qualitatively different from that of the vessels in voluntary muscle.

Dale [1913] has shown that stimulation of the splanchnic nerve after intravenous injection of ergotoxine will cause a fall in blood-pressure and interprets this as evidence of the presence of vaso-dilator fibres in this nerve. Fig. 7 shows that intra-arterial adrenaline will produce dilatation in the mesenteric vessels if ergotoxine has been previously given, and here again the initial pressure is below the "critical level" of Cannon and Lyman [1913]. It is apparent that dilatation is still possible with a general blood-pressure below 60 mm. Hg in Fig. 7 B, and therefore its failure to appear in Fig. 7 A is not due to initial maximal dilatation.

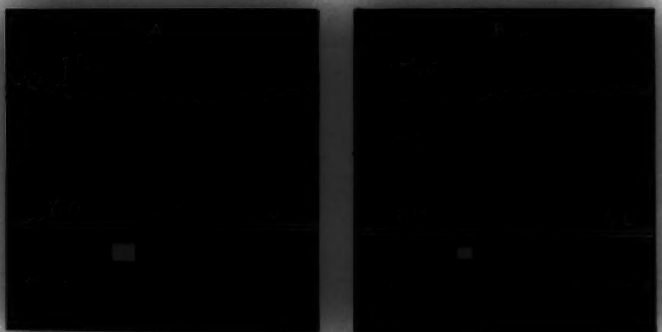

Fig. 8. Blood-pressure and venous flow from skin of hind leg. A. 0·02 c.c. adrenaline 1×10^{-6} into iliac artery. B. 0·05 c.c. adrenaline 1×10^{-7} into iliac artery. The figures above the drop record indicate flow in c.c. per min.

Skin vessels. The minute vessels of the skin appear to respond directly to very small doses of adrenaline in the same way as do those of the intestine, but previous work in which plethysmographic methods were used indicates that the constriction in skin is more intense, in that it does not give way to dilatation when the blood-pressure rises [Clark, 1930]. In the case of skin, however, it is difficult, when recording changes of flow only, to determine to what extent these changes are due to alterations in calibre of the arterio-venous anastomoses described by Grant and

Bland [1931] and to what extent they are due to variations in capillary calibre. Taking into consideration previous investigations by direct observation [Hoskins, Gunning and Berry, 1916; Lewis, 1927] and by plethysmographic methods [Clark, 1930], however, the conclusion may be drawn that adrenaline in amounts which have any detectable action causes constriction of the minute skin vessels. Fig. 8 shows the effect on venous outflow from the skin of the hind leg of the intra-arterial injection of adrenaline. In no case was an increased flow found.

If sympathetic vaso-dilator fibres to the vessels of the skin exist, it should be possible to unmask them by injecting adrenaline after giving ergotoxine. In several experiments in which this was done there was no increased rate of flow through the skin vessels; the dose of ergotoxine given was 2·5 mg. per kg., and following this 0·05 c.c. of adrenaline diluted 1 in 1 million injected intra-arterially gave a transient fall in blood-pressure varying from 5 to 10 mm. Hg. The ergotoxine caused considerable slowing of the blood flow through the skin.

Sympathin. Since the greater part of the experimental work described above was done, Cannon and Rosenblueth [1933] have produced direct evidence for the presence of a substance, Sympathin E, which is released by stimulation at vaso-constrictor sympathetic nerve endings. Evidence is also given of a similar substance, Sympathin I, formed by dilator or inhibitor nerve endings. These findings amply explain the dual effect in the vessels of muscle of an intra-arterial injection of adrenaline which presumably releases both Sympathin E and Sympathin I; it seems, however, that the dilator endings have a lower threshold to adrenaline than do the constrictor.

Experiments were devised to determine the possibility of showing the presence of Sympathin I. Two cats were anæsthetized with chloralose and one of these (*A*) was prepared as previously described to record the blood flow from a group of muscles in the hind leg. In the second cat (*B*) the suprarenal glands were tied off with a pedicle ligature and a portion of the portal vein laid bare; the blood-pressure was recorded in the usual way. Ergotoxine was then given (2·5 mg. per kg.), and when the blood-pressure had settled at its new level adrenaline was injected. Samples of blood were taken from the portal vein (*a*) before giving adrenaline, and (*b*) at various intervals after adrenaline; 0·1 c.c. of these samples was injected into the iliac artery of cat *A*. It was found that the samples (*a*) had no effect on muscle blood flow and the samples (*b*) taken within 10 min. of giving adrenaline were also usually without effect, but samples taken between 10 and 15 min. gave a dilator response (Fig. 9); if, however, the

adrenaline had caused a rise in blood-pressure in cat *B* this dilator response was followed by constriction (Fig. 10). A simple calculation and the lack of response to earlier samples (*b*) showed that the amount of adrenaline given to cat *B* was not responsible for this effect, and the conclusion is that a dilator substance had been released from sympathetic inhibitor and dilator nerve endings in cat *B*.

Fig. 9.

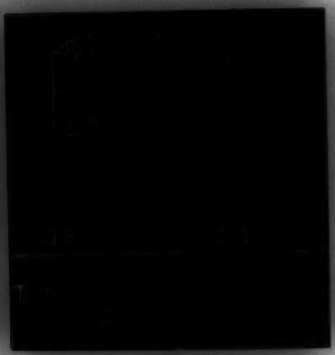

Fig. 10.

Fig. 9. Blood-pressure and venous flow from muscle of hindlimb. At A, 0·1 c.c. blood intra-arterially, from second cat given 0·4 c.c. adrenaline (1×10^{-4}) intravenously after ergotoxine; blood drawn from portal vein 13 min. after adrenaline given. The figures above the drop record indicate flow in c.c. per min.

Fig. 10. Similar to Fig. 9, but adrenaline in second cat had caused a rise in general blood-pressure despite ergotoxine. The figures above the drop record indicate flow in c.c. per min.

Discussion.

The double action of adrenaline, dilator and constrictor, has been discussed by Dale and Richards [1918] and is well shown as the result of a single injection in the volume record of a skinned limb in Fig. 15 D of their paper; the fact there shown that the volume of the muscle alters in a manner similar to the venous outflow clearly indicates that the increased outflow is not due to constriction of vessels squeezing more blood out of the muscle but is a true expression of vaso-dilatation. Dale and Richards are of the opinion that both dilatation and constriction have their site in the capillaries, and this view is supported to some extent

by the result described above of giving repeated injections of adrenaline (Fig. 3); if in this case dilatation took place in the capillaries but constriction in the arterioles a second injection during the action of the first would merely intensify the double effect, and further arteriolar constriction, in the absence of a rise in blood-pressure, would prevent any increase in venous outflow. Again, if we assume, contrary to the evidence of Dale and Richards, that small doses of adrenaline dilate arterioles and constrict capillaries simultaneously, it is highly improbable that a second injection by increasing arteriolar dilatation (and presumably further constricting capillaries) would cut short the constrictor effect in the capillaries by mere passive dilatation in view of the pressures which Lewis [1927] has shown constricted capillaries can withstand. It is true that Lewis's observations refer to the minute vessels in human skin and therefore may not be directly applicable to capillaries in muscle, but in the latter vessels adrenaline dilatation has been obtained when the arterial pressure was as low as 50 mm. Hg (Fig. 5), a pressure against which Lewis has shown that even dilated capillaries can constrict. The fact that vaso-dilatation due to adrenaline occurs at this low pressure does not support the view of Cannon and Lyman [1913] that the effect of adrenaline depends on the state of the vessels in any given case.

It does not seem possible in the present state of knowledge to harmonize the observation that repeated small injections of adrenaline produce prolonged or repeated dilatation (Figs. 3 and 4 B) with that of Dale and Richards [1918] and of Burn [1932] that a low concentration of adrenaline added to blood perfusing a limb will restore constrictor tone in the minute vessels. Some experiments as yet incomplete, however, suggest that an explanation of the apparent discrepancy may be found, but the problem must be left over for further investigation.

The difference in reaction between intestinal vessels and the vessels in muscle cannot be due to the absence in the former of the means for active dilatation, for this latter reaction can be brought about by adrenaline after ergotoxine; there is evidence that sympathetic vaso-dilator fibres are relatively few in the splanchnic area [Cannon and Rosenblueth, 1933], and it may be that their threshold to adrenaline is also relatively higher than in muscle. In the skin it does not seem possible to show the presence of sympathetic dilator fibres by the technique adopted here; conditions are probably complicated by the presence of arterio-venous anastomoses [Grant and Bland, 1931], and it is impossible to tell by means of blood-flow methods whether changes in flow caused by adrenaline are due to variations in calibre of these anastomoses or of capillaries. It does appear, however, that adrenaline, in the smallest

amount which has any effect, always causes a diminution in blood flow through the skin and that this response is not reversed by previous administration of ergotoxine.

Summary and conclusions.

The action of minute amounts of adrenaline given intra-arterially on the blood flow through skeletal muscle, intestine and skin in the cat has been investigated.

The response of muscle vessels to a single injection is of a twofold nature, first dilatation and then constriction, although cases are recorded in which only constriction was seen with the smallest dose of adrenaline that would give any response. This response to adrenaline in the vessels of skeletal muscle is not qualitatively altered by any change in body temperature likely to be found under physiological conditions. Evidence is given of the release of a dilator substance analogous to Cannon's Sympathin I when only inhibitor or dilator sympathetic endings are stimulated by adrenaline, stimulation of constrictor endings being prevented by ergotoxine.

The smallest effective amount of adrenaline always produces a diminished flow of blood through the intestine and skin. In the intestine, but not in the skin, this response is reversed by previous injection of ergotoxine.

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"INHIBITION" IN MEDULLATED NERVE.

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Feng and Hill [1933] have recently studied the heat production of frog's nerve during the prolonged application of a stimulus of known frequency. They have shown that with a relatively low frequency (50–100 shocks/sec.), after a certain time a constant rate of production of heat is arrived at; this can be maintained for a very long time but diminishes, rapidly at first and then gradually, when excitation ceases, finally returning to the level corresponding to rest. With a high frequency, however (500–1000 shocks/sec.), a steady production of heat cannot be obtained; the rate at which heat is produced increases rapidly at the beginning of excitation, reaches a maximum, then decreases in spite of the maintenance of the stimulus. If, during this state of apparent fatigue, a stimulus of high frequency is replaced by one of low frequency, an immediate increase is observed in the rate of production of heat: the nerve, which no longer responds to the excitation of high frequency, is nevertheless capable of responding to an excitation of low frequency. This phenomenon, which was further discussed by Hill at the 1933 meeting of the American Association in Chicago [1934], is the subject of the present investigation.

Technique.

The apparatus used was similar to that described by Hill [1932] and by Feng and Hill [1933]. The thermostat, however, had been modified as follows. The wooden box, which forms its external cover, instead of being placed directly in the room, was enclosed in a second wooden box covered inside with zinc sheet. The air space, separating the two boxes, was maintained at a constant temperature by an electrical heating arrangement automatically regulated. The temperature of the air space

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could easily be maintained constant within 0·1° C. This made unnecessary the maintenance of a constant temperature in the room as a whole, and increased considerably both the thermal stability and the ease of working.

The deflection of the galvanometer was read directly on a graduated scale without photographic recording. The scale was placed 4·5 metres from the galvanometer and the spot could easily be read to the nearest millimetre. Readings were made every 15 sec. during the first few minutes following the application or removal of a stimulus, and every minute later on, when an approximately steady state had been reached.

As stimuli, condenser discharges were employed at a frequency determined by the speed of rotation of a commutator [Hill, 1932]. Since the frequency had to be varied abruptly, two commutators were used running at different speeds. The reversal of the key allowed an instant transfer from one frequency to the other. One of the commutators was used for low frequencies (50–100 shocks/sec.), the other for higher frequencies (usually 500–1000 shocks/sec.). The condenser discharges themselves were exactly the same whatever the frequency, since they depended only upon the capacity of the condenser, which was the same in every case, and the resistance through which it discharged, which was also the same.

Frogs' sciatics were used, from large Hungarian *R. esculenta*, eight nerves in each experiment.

In general, in order to avoid polarization at the stimulating electrodes, excitation was effected by alternating charge and discharge of the condenser; thus excitation occurred alternately at one electrode and the other. In certain experiments, however, condenser discharges alone were employed, the stimuli being all in one direction, the cathode being at the electrode which was nearer to the thermopile; in certain other experiments there were two pairs of electrodes, each pair being used for one of the two frequencies. A suitable manipulation of the keys allowed an instant transfer from one mode of stimulation to the other (alternating or "one-way") and the use, at will, of one or other pair of electrodes. Actually three electrodes only were necessary, *A*, *B* and *C*, in that order pointing towards the thermopile. The distances were: *A* to *B*, 3 mm.; *B* to *C*, 12–15 mm.; *C* to thermopile, 18 mm. The distance from *B* to *C* was so great that stimulation at *A* or *B* could scarcely have affected the nerve at *C*. For the lower frequencies *A* and *B* might be employed, *B* being the cathode: for the higher frequencies, *B* and *C*, *C* being the cathode. *C* was actually the block of silver ordinarily serving to prevent the passage of heat from the stimulating electrodes to the thermopile [see Hill, 1932,

p. 113, Fig. 3]. The usual electrodes *A* and *B* will be described as the "upper" electrodes.

The capacity of the condenser used for stimulating, the potential to which it was charged, and the resistance short-circuiting the nerve, were chosen according to the principles discussed by Hill [1932]. In view of the high frequency of excitation employed, it was necessary to ensure that the condenser discharges were complete in the short interval of contact on the commutator. Unless this precaution be taken the shocks making up the stimuli will not be independent of the frequency. The capacity required can be determined by calculation, assuming for example that not more than 1 p.c. of the energy of the condenser should be undischarged at the moment when the discharge is cut short.

The following test was made. In the ordinary excitation circuit, namely, nerve in series with 5000 ohms, short-circuited by 1000 ohms, the latter was replaced by a resistance wire (932 ohms) wound non-inductively on a thermopile. The system could be connected to one or other of the commutators. The deflection of a galvanometer connected to the thermopile on which the hot wire was wound allowed one to measure the energy of the current through the wire. With a known frequency of charge and discharge one could determine the maximum capacity which would allow discharge to be complete to any required degree in the time available between separate contacts. Provided that the contacts are clean and not too oily, and the brush in good order, the rules are obeyed.

With a capacity of 0·105 μF the readings obtained with the commutator and those with a constant current calculated to give the same energy were compared. Between 100 and 1200/sec. the difference was never more than 4 p.c. At a frequency of 1950 the difference had risen to 12·5 p.c. In the experiments reported the stimuli were always as follows: capacity, 0·105 μF, charged to 14·4 volts, 5000 ohms in series with nerve, nerve and resistance short-circuited through 1000 ohms: time of half discharge, 0·06 σ; of 99 p.c. discharge, 0·4 σ. These shocks were well super-maximal. In general the frequency was kept below 1200/sec.

In order to keep permanent control during each experiment on the effectiveness of the stimulus, the following arrangement was finally adopted. The 1000 ohms shunt ordinarily used was replaced by a hot wire wound on a thermopile, the resistance of the hot wire being 932 ohms. At any point during an experiment a reading of a microammeter connected to the thermopile on which the hot wire was wound, is a measure of the energy in the stimulus. During each experiment several readings were made on the microammeter; the readings were always consistent and provided evidence of the efficiency and constancy of the stimuli.

The experiments were all made at a temperature between 21·2 and 22·7° C.

Sensitivity.

The apparatus was calibrated by observing the steady deflection produced by a series of regular condenser discharges applied between the heating electrodes after the nerve had been made inexcitable by prolonged

treatment with nitrogen. The sensitivity was practically constant, the following being the mean value: 1 mm. of steady deflection corresponded to $1{\cdot}81 \times 10^{-8}$ cal./sec., or to $12{\cdot}9 \times 10^{-8}$ cal./g./sec.

Heat leak.

By reason of the high frequency of the stimuli used, it was feared that the heat might leak from the stimulating electrodes along the nerve into the thermopile in spite of the usual precaution of a block of silver placed between the two. In a certain number of experiments, therefore, the effect of the various stimuli employed between their usual electrodes was tested on nerves previously killed by long residence in nitrogen. In no case could one detect any important leakage of heat. The maximum effect observed corresponded to 8 p.c. of the heat found in the same experiment with the nerves stimulated alive, and even then only with a very high frequency, namely 2000/sec. In most cases the heat leak was quite negligible.

RESULTS.

In the first experiments the upper electrodes only (*A* and *B*) were used with stimuli alternating in direction in order to avoid polarization. Each individual shock, whether the frequency was low or high, had the same energy and time relations of discharge and was chosen to be approximately optimal for a nerve at rest.

The phenomenon observed is as follows. When, by means of an excitation of long duration and of relatively low frequency (50–100/sec.), a steady state of heat production by the nerve has been established, if the frequency is raised abruptly (*e.g.* to about 1000/sec.) an abrupt diminution is observed in the production of heat, the galvanometer moving rather rapidly in the negative direction. The initial heat production is diminished, or nearly abolished, though of course the recovery heat production from previous stimulation remains and follows its normal course. The nerve appears at first to be "fatigued." If, however, to the nerve apparently fatigued one substitutes in place of the excitation of high frequency the previous excitation of low frequency, one observes at once a production of heat at the previous higher rate and a return to the steady state characteristic of the original low-frequency excitation. The nerve, which seemed to be "fatigued" when tested by a high-frequency stimulus, is not fatigued at all when tested by a low-frequency stimulus. The same series of phenomena can be repeated many times. Some experiments have

lasted for more than 3 hours, during which a steady state of heat production has been maintained under excitation of low frequency, interrupted by abrupt falls in the rate of heat production following the application of a stimulus of high frequency (Fig. 1).

It is important, however, to note that the high-frequency stimulus produces a maximal thermal response when applied to a resting nerve. With a stimulus of given short duration to a resting nerve, the heat increases with frequency up to very high values of the latter, as is shown by the usual heat-frequency relation described by A. V. Hill and his collaborators in several previous communications.

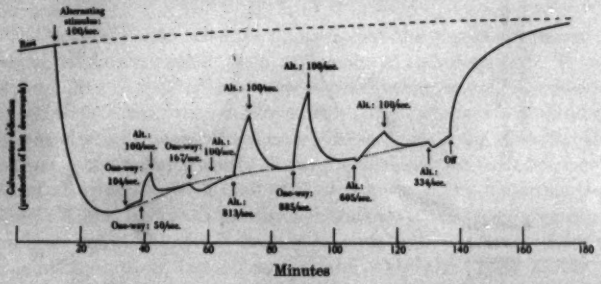

Fig. 1. Heat production of frog's nerve at about 22° C. during continuous excitation at various frequencies for 125 min. Experiment of May 15, 1933. Rate of heat production downwards as galvanometer deflection without analysis. Alternating or one-way stimuli as shown. The broken line interpolated between the beginning and the end of the experiment is a base line corresponding to the rate of heat production of the resting nerve. The dotted line corresponds to the steady and nearly constant rate of heat production during continuous stimulation at 100/sec. For further description see text.

The facts have been verified in fifteen experiments. One may begin by exciting the nerve during a state of rest by a high-frequency stimulus (for example, May 8, 10 and 26 respectively, 1284/sec., 920/sec., 920/sec.). There is an immediate and large positive deflection of the galvanometer corresponding to an intense production of heat by the nerve. At the end of 2 min., in place of the high-frequency stimulus effective on the resting nerve, a low-frequency stimulus is applied; the nerve continues to produce heat, though at first at a lower rate, and in the appropriate time reaches a steady state. The substitution now of the high-frequency for the low-frequency stimulus immediately breaks down the steady state

and causes not an increase but an abrupt decrease in the rate of production of heat. A return to the low-frequency stimulus in place of the high causes a gradual re-establishment of the previous steady condition.

The sudden diminution in the rate of heat production caused by substituting a high for a low frequency does not represent a complete

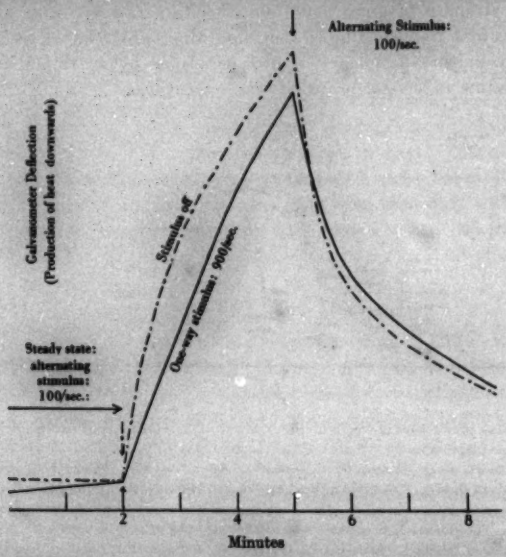

Fig. 2. Experiment of May 17,1933. Comparison of the effect obtained during a steady state of heat production by a nerve stimulated continuously at a low frequency, 100/sec., (a) by stopping the stimulus altogether (broken line) and (b) by changing the stimulus to one of high frequency, 900/sec. Rate of heat production downwards as galvanometer deflection without analysis. Note that the high-frequency stimulus does not produce complete cessation of response.

cessation of response, at least in all the fibres. If, during the condition of diminished rate of heat production caused by applying a high-frequency stimulus as described, one stops the stimulus altogether, one immediately observes a rather more rapid fall in the rate of production of heat, as shown by an acceleration in the movement of the galvanometer towards its resting position. In one experiment (May 17) deflections in the negative

direction were compared during a steady state of stimulation at 100/sec.: (*a*) when excitation was stopped altogether, and (*b*) when a low-frequency stimulus was replaced by one at 900 shocks/sec., the interval in either case being 3 min. For complete cessation the deflection was 74 mm., for high-frequency excitation it was 67 mm. (Fig. 2). A little later in the same experiment, again for 3 min., cessation of excitation gave a negative deflection of 71 mm., a stimulus of 612 shocks/sec., a negative deflection of 55 mm. Similarly, the rise in the heat production on returning to the stimulus of low frequency is greater after an interruption in the stimulus than after an excitation at high frequency. The latter, therefore, does not cause a complete cessation of response, except perhaps at very high frequencies.

One naturally asks whether the fact that the shocks alternated in direction might not explain their inadequacy at high frequency. To test this possibility, in spite of the danger of polarization, in a certain number of experiments stimuli were employed consisting of shocks in one direction only, the electrode nearer the thermopile being made the cathode. The effect was compared with that of alternating shocks of the same frequency. First of all it was verified that with excitations of low frequency (50–167/sec.) it is a matter of indifference whether alternating or one-way stimuli are used. The steady state of heat production is not modified at all, or at most to an insignificant extent, when alternating shocks at 100/sec. are replaced by one-way shocks at the same frequency. In order, therefore, to avoid polarization alternating shocks were used at the low frequency as conditioning stimuli to obtain the steady state.

In one experiment (May 11) there were applied successively, during a steady state with an alternating stimulus at 100/sec., and replacing that stimulus, (*a*) an alternating stimulus of 807/sec., (*b*) a one-way stimulus at 884/sec., and (*c*) an alternating stimulus at 807/sec. The alternating stimulus gave at first in both cases a slight extra production of heat followed by a very rapid diminution. The one-way stimulus gave immediately a rapid diminution. In all three cases a return to excitation at 100/sec. gave an immediate positive deflection and a return to the steady state.

In another experiment (May 15) an alternating stimulus of 813/sec. and a one-way stimulus of 885/sec., when substituted for a steady stimulus of 100/sec., were immediately followed by a large negative deflection more rapid in the case of the one-way stimulus. A return to the stimulus at 100/sec. brought an immediate positive deflection and a return to the steady state. The same result was obtained in another experiment

(May 17). It seems therefore that high-frequency shocks in one direction produce the same phenomenon as alternating shocks of the same frequency, and that it is not the alternation in direction of the latter that is the cause of their inadequacy. Indeed, the diminution of heat is slightly greater when the shocks are in one direction only. This may be due to an effect of polarization adding on to the effect of the high frequency, but it is at least possible that with alternating shocks at electrodes *A* and *B*, when the frequency is high, the impulses set up at *A* are unable to pass electrode *B* and so to reach the thermopile. Thus, alternating shocks at 1000/sec. at *A* and *B* may effectively be no more than one-way shocks at 500/sec. at *B*. If so, the high-frequency effect would be more obvious with one-way shocks at 1000/sec. at *B*.

In one experiment (May 26) the following result was observed. During the state of diminished heat production caused by substituting a one-way stimulus of 1000/sec. for an alternating stimulus of 100/sec. during a steady state caused by the latter, an alternating stimulus of 1090/sec. was applied. The immediate effect was a very slight positive deflection followed by a negative deflection similar to that preceding it. It seems, therefore, that one-way stimuli are at least as effective in producing the type of "inhibition" referred to as alternating stimuli.

Another possible explanation of the phenomenon was a local modification of the nerve set up at, or between, the electrodes by the low-frequency stimulus. This local experimental modification of the condition of the nerve might affect it in its response to the high-frequency stimulus between the same electrodes. To test this possibility, in a certain number of experiments two pairs of electrodes were utilized as described above, *A* and *B* or *B* and *C*. First of all it was verified that the two pairs of electrodes were identical for the establishment of a steady state of heat production with an excitation of low frequency. In one experiment (May 18), during a steady state of activity set up by an alternating stimulus at the upper electrodes *A* and *B* (frequencies 100/sec.), the nerve was excited at the lower electrodes *B* and *C* at frequencies of 611/sec. and 912/sec. In both cases an abrupt negative deflection was produced followed by a positive deflection and a return to the steady state when the low-frequency stimulus at the upper electrodes *A* and *B* was substituted for the high-frequency stimulus at *B* and *C*. In the same experiment a stimulus of 912/sec. at the upper electrodes *A* and *B*, substituted for 100/sec. at the same electrodes, produced the same phenomenon, the negative deflection being more rapid than when *B* and *C* were employed. Some local effect, therefore, may occur, but it is not the cause of the phenomenon.

In the experiment of May 22, during a steady state with a stimulus of 48/sec. at the upper electrodes *A* and *B*, successive stimuli were applied as follows, all at 605/sec.: (1) at *A* and *B*, (2) at *B* and *C*, (3) at *A* and *B*. In all three cases, owing to the rather lower frequency, a very short positive deflection was observed followed by a large negative deflection. The same results were obtained in the experiments of May 24 and May 26. The phenomenon, therefore, cannot be explained by a local modification of the nerve at or between the electrodes of the conditioning stimulus. The distance from *B* to *C* was 12–15 mm., so it is very unlikely that shocks of low frequency at *B* could directly affect the nerve at *C* sufficiently to block it to subsequent high-frequency stimulation. The effect found in high-frequency stimulation at *C* must have been conditioned by low-frequency impulses previously reaching *C* from above, and not by the direct effect of the shocks.

If, during a steady state of heat production, due to prolonged excitation at low frequency, more rapid stimuli of various frequencies are applied, a graduated effect is obtained in the modification of the heat production. In one experiment (May 8), during a steady state produced by a stimulus of 112/sec., a stimulus of 705/sec. replacing it, caused a positive deflection followed by a large negative deflection. A stimulus of 1008/sec. produced a slight positive deflection followed by a negative deflection more rapid than the preceding. A stimulus of 1570/sec. produced a negative deflection immediate and abrupt. In another experiment (May 15) analogous results were obtained at frequencies of 305/sec., 605/sec., 813/sec. The same was found in eight experiments in which, not only the frequency was varied, but also the electrodes and the mode (one-way or alternating) of excitation. All the effects intermediate between a production of heat and its almost complete suppression can be obtained when a prolonged excitation at low frequency is changed to an excitation of high frequency. In every case the return to the excitation of low frequency causes a re-establishment of the previous state.

The duration of the preceding excitation of low frequency also has an influence on the thermal response of the nerve to a stimulus of high frequency. In one experiment (May 22) after 30 min. stimulation at 48/sec., the steady state of heat production not having been quite reached, an excitation of 605/sec. was applied. A small positive deflection was observed which lasted for 3 min. and was succeeded by a negative deflection. A return to the 48/sec. then caused a restoration of the previous state of steady heat production. After a total of 80 min. of low-frequency excitation the stimulus of 605/sec. was again applied, this time with an

immediate fall in the production of heat. In another experiment (May 23) a stimulus of 906/sec. maintained for 2 min., after 12 min. of stimulation

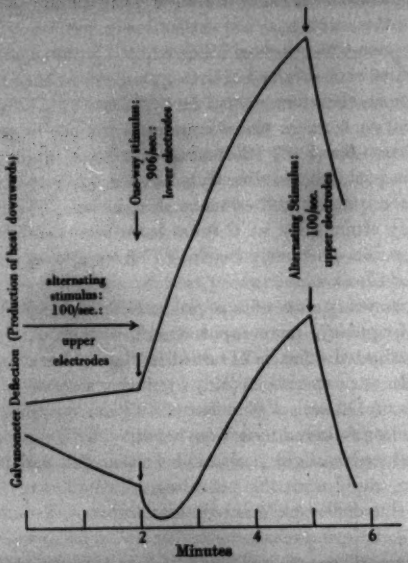

Fig. 3. Influence of the duration of the conditioning low-frequency stimulus (100/sec.) on the heat production of nerve when the frequency is suddenly raised to 906/sec. Experiment of May 24, 1933. The lower curve shows the application of the high-frequency stimulus to a nerve previously stimulated for 12 min. only at 100/sec., the steady state of heat production corresponding to this low frequency not yet having been attained. An immediate extra production of heat occurs, followed after 30 sec. by a diminution. The heat production rises again on returning to the lower frequency. The upper curve corresponds to the high frequency applied to the same nerve previously stimulated for 31 min. at the lower frequency, the steady state of heat production corresponding to the low frequency having already been reached. The diminution in heat production is immediate but the curve rises again when the frequency is again lowered. The low and the high-frequency stimuli were applied at two different pairs of electrodes, as described in the text, the conditioning low-frequency shocks being at electrodes more distant from the region of the nerve in which the heat production was measured. Heat production downwards as galvanometer deflection without analysis.

at 103/sec. which had not yet led to the establishment of a steady state, produced an immediate negative deflection of 60 mm. After a total of 30 min. of stimulation at 103/sec., a steady state of heat production being

now established, the same high-frequency excitation now gave an immediate negative deflection of 82 mm. Analogous results were obtained in the experiments of May 24 and 26 (Fig. 3). The production of heat by the

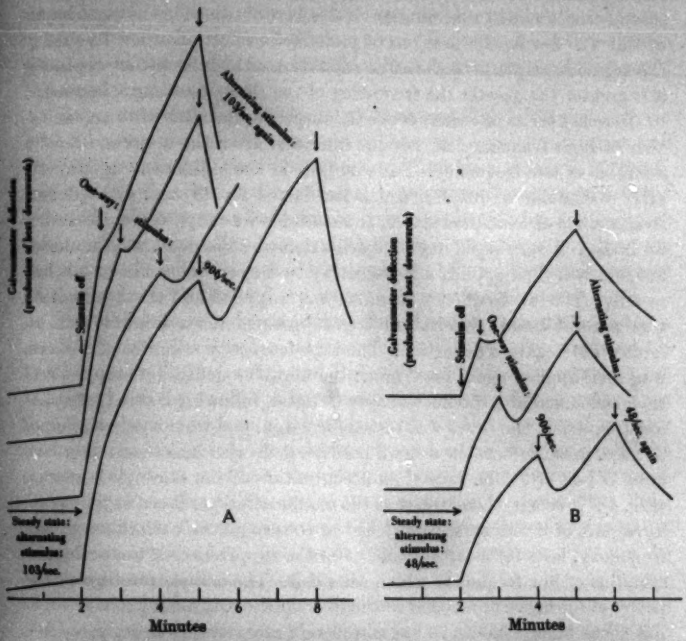

Fig. 4. Effect of an excitation of high frequency (906/sec.) on the heat production of nerve rested for various intervals after a previous prolonged stimulation leading to a steady state at low frequency. A, conditioning frequency 103/sec.; B, conditioning frequency 48/sec. Intervals of rest: A, $\frac{1}{4}$, 1, 2 and 3 min.; B, $\frac{1}{4}$, 1 and 2 min. Note that in B the response to the high-frequency stimulus is greater after a conditioning stimulus of low frequency than after one of a rather higher frequency. Heat production downwards as galvanometer deflection without analysis. Experiment of May 23, 1933. For discussion see text.

nerve, therefore, is more rapidly and intensely diminished as the result of an excitation of high frequency, when the duration of the low-frequency excitation which it replaces has been greater.

The frequency of the initial slow stimulus plays a similar part in the

response to a high-frequency stimulus following it. In the experiment of May 23 an excitation of 906/sec., lasting for 2 min., followed first an excitation of 100/sec. lasting for 12 min., then an excitation of 48/sec. lasting for 12 min. In the first case the negative deflection was 60 mm., in the second case it was 40 mm. Similar results were obtained in the experiment of May 22. For equal durations of prolonged excitation at low frequency the negative response to the same excitation of high frequency replacing it is greater the greater the frequency of the slow preceding stimulus.

In a last series of experiments the response was studied to an excitation of high frequency at various intervals after the suppression of a stimulus of low frequency. For example, in the experiment of May 23, after a stimulus of 103/sec. which had lasted for 45 min. and led to a steady state of heat production, the stimulus was suppressed altogether for 30 sec. A very rapid negative deflection was observed. A stimulus of 906/sec. was then applied and the negative deflection continued but less rapidly. The low-frequency stimulus was reapplied and the steady state was re-established. Excitation was suppressed for a minute with an immediate negative deflection. The high-frequency stimulus of 906/sec. was then applied again for 2 min.: the negative deflection stopped and then continued at a diminished speed. Later, following a steady state at low frequency, the nerve was rested for 2 min. and then an excitation of 906/sec. produced at first a small positive deflection and then a negative one. A rest of 3 min. caused an accentuation of the same phenomenon (Fig. 4). It seems, therefore, that the modification produced in the nerve, in respect of which it fails more or less to respond to a stimulus of high frequency, lasts for a certain time after the suppression of the prolonged stimulus of low frequency which caused it. The complete return of the power of the nerve to respond to a high-frequency stimulus probably does not occur before the nerve has completely recovered from its preceding steady excitation.

Discussion.

The experiments reported show that prolonged excitation of a nerve by shocks of low frequency renders it incapable, to a greater or less degree, of responding to a stimulus of high frequency. This is not a question, however, in any ordinary sense of "fatigue." The nerve, which is no longer excitable by, or gives only a small response to, a stimulus of high frequency, responds to a stimulus of low frequency by a production of heat which is constant and may be continued for a very long time, or almost indefinitely. It seems rather that the nerve during the course of prolonged excitation at low frequency is put into a state in which it is

"inhibited" or partly inhibited, by a stimulus of high frequency. The state in which inhibition, instead of excitation, occurs when a rapid stimulus is applied, is reached only gradually, after a time of stimulation at low frequency of the order of 10, 20 or 30 min. The duration required depends upon the frequency of the slow excitation employed. The inhibitory effect is shown in all degrees as one increases progressively the frequency of the conditioning stimulus, its duration, and the frequency of the rapid stimulus tested.

Experiments of Hill [1932] and of Feng and Hill [1933] have shown that there is no simple relation between the frequency of excitation and the heat produced by the impulses set up. Another variable is involved, namely, the duration of the stimulus employed, and it now seems that the state of the nerve as affected by previous stimulation also must be taken into account. The heat per impulse is a function, not only of the frequency of excitation, but of the duration of previous stimulation and of the previous history and the state of recovery of the nerve.

One naturally asks whether the effect observed may not be due to a modification in the duration of the refractory period. Forbes and his collaborators [1923], Gasser and Erlanger [1925], and numerous other authors, have shown that if impulses follow one another in a nerve, at intervals short enough to make it necessary for them to travel in fibres incompletely recovered, the velocity of propagation and the size of the action current are modified: the refractory period is increased, the speed of propagation reduced, the size of the electric changes diminished. Such experiments have been concerned with the propagation of two impulses only, or of a series of impulses far shorter than we have considered in the present experiments.

The lengthening, however, of the refractory period, after a long interval of excitation at low frequency, is not sufficient alone to explain the "inhibition" to stimuli of high frequency. While admitting that a greater proportion of the shocks in the high-frequency stimulus would fall, in consequence of the lengthening of the absolute refractory period, within an interval of inexcitability of the nerve, it is difficult to understand why the response to an excitation of high frequency should not be (as it is in a nerve at rest) at least as great as to the excitation of low frequency. It seems, as Hill [1934] remarks, and as Verworn [1914] supposed for a series of shocks leading to Wedensky inhibition, that a stimulus *B* falling in the absolute refractory period of an impulse *A* must extend the refractory period, *i.e.* must render the nerve inexcitable by a following stimulus *C*, although *C* following *A* alone would have produced a response.

Fröhlich also (referred to by Lucas [1911]) supposed that "recovery" may be hindered by stimuli falling within the absolute refractory period: though Lucas himself could find no trace of such a relation with three stimuli applied to a previously resting nerve. The state in which the relation seems actually to occur is acquired by a nerve during slow steady stimulation at a rate to which it can respond indefinitely, and which does not therefore in any ordinary sense fatigue it. In a resting nerve, with a stimulus of short duration, the higher the frequency the greater the response.

This "inhibition" recalls in certain respects the phenomenon described under the name of Wedensky inhibition. When a muscle-nerve preparation has been modified by fatigue, or by a narcotic, stimuli of high frequency to the nerve no longer produce muscular response. The explanation generally given of this phenomenon involves the modification of the state of the neuro-muscular junction. Wedensky [see Lucas, 1911, p. 84] showed, moreover, that similar inhibition may occur in nerve alone, with local impairment of conduction. Tsai [1931] also has shown that a narcotized portion of nerve will allow impulses derived from a muscular sense organ to pass at a limited frequency but will arrest impulses of a higher frequency. In the present case the conditioning cause can scarcely be a local block since previous slow excitation at a point 12–15 mm. above the point tested renders the nerve liable to "inhibition" on high-frequency stimulation. H. and P. Davis [1932] have shown that the tension exerted by a muscle is a maximum for stimuli of low frequency and is diminished when the frequency is raised. These phenomena are probably all of the same nature.

It seems then, that when a nerve is subjected to prolonged steady non-fatiguing stimulation of moderate frequency, a condition is gradually set up in which a stimulus of high frequency produces a state of inhibition. During, and for some time after, this state of steady and constant excitation (which is presumably at least as representative of the normal physiological state as one of complete rest) the process of "recovery" from the refractory condition following a shock is different from that observed in the case of an isolated impulse, or during stimulation of short duration of whatever frequency applied to an otherwise resting fibre.

Summary.

When a nerve is subjected to prolonged stimulation of low frequency (up to 100 shocks/sec.) it finally reaches a steady state in which the rate of heat production is constant. In this state, although in no ordinary sense fatigued, it is largely incapable of responding to an excitation of high

frequency of the order of 1000 shocks/sec. It remains, however, capable of responding by the same steady heat production on returning to the stimulus of low frequency. If, during a state of rest, the nerve is subjected to the same high-frequency excitation, it now gives an immediate maximal response. The phenomenon seems to be of the same nature as Wedensky inhibition, but it occurs in the nerve trunk.

The effect is not due to any local modification of the nerve at the exciting electrodes. It appears at a considerable distance from the point at which the conditioning stimulus of low frequency is applied, as is shown by the fact that the same failure to respond to a high frequency may occur at a distant electrode. The effect is the same whether the stimuli alternate in direction or are all "one-way."

All degrees can be observed between complete inhibition and a steady production of heat when the frequency of the secondary stimulus (the rapid one) is varied.

The fall in the response on the application of the higher frequency is greater and more rapid the higher the frequency of the basic conditioning stimulus and the longer the time of its application. When the low-frequency conditioning stimulus is stopped and an interval of recovery allowed, the response to the high-frequency stimulus is greater the greater the interval of intervening rest.

It is difficult to give an explanation of the phenomenon on the basis of what is known about the passage of a single impulse, or of a few isolated impulses, in an otherwise resting nerve. As the result of prolonged steady activity, not so intense, however, as to lead to fatigue, the processes of "recovery" from the refractory state following a shock are modified, so that a continuously refractory condition may be set up by shocks succeeding one another at sufficiently short intervals.

My warmest thanks are due to Prof. A. V. Hill for suggesting the subject of this work and for providing the material and the counsel necessary for its realization. My thanks are due also to Mr J. L. Parkinson for his help and advice.

APPENDIX.

By A. V. HILL.

The state of "inhibition" described in the preceding paper is not propagated away from the stimulating electrodes, as is shown by the following experiment. A thermopile was prepared with four electrodes, A, B, C and D, the distances being as follows: $AB = 2{\cdot}2$ mm., $BC = 16$ mm.,

CD = 3·4 mm., D to nearer end of thermopile = 18 mm. A high frequency stimulus (1000/sec.) initially producing a maximal response was applied at A and B until the response was largely reduced; without stopping the stimulus at A and B a low frequency stimulus (100/sec.) was then applied at C and D and a good response was obtained.

Another experiment was made as follows. A short (16 sec.) high-frequency stimulus at A and B initially caused a much greater response than a similar low-frequency stimulus at C and D. When the latter had been continued for some time, the former applied for 16 sec. during the continuance of the latter always gave a slight positive effect, showing (*a*) that there was not complete failure at A and B, and (*b*) that nothing like "inhibition" was being propagated from AB to CD. If, however, the low frequency at CD was turned off while the high frequency at AB was turned on, there was a negative response, showing that the high frequency was now producing a smaller effect than the low frequency, therefore a much smaller effect than it did initially.

It seems, therefore, that the "inhibition" referred to by Bugnard is connected with the process of excitation, each element of the high-frequency stimulus causing, or maintaining, a higher threshold or a prolonged refractory period. The condition in which this "inhibition" may be observed on high-frequency stimulation can be produced by a steady stream of impulses propagated from a distant point. The "inhibition" itself, however, is local. One cannot "inhibit" low-frequency impulses starting at CD by high-frequency stimulation at AB; though one can block (at CD) low-frequency impulses starting from AB by high-frequency stimulation at CD.

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THE ANAEROBIC BREAKDOWN OF CARBOHYDRATE IN THE ISOLATED VENTRICLE OF THE FROG.

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It has been shown by Clark, Gaddie and Stewart [1931] that the isolated heart of the frog beating in air or oxygen derives at least part of its energy from non-carbohydrate sources. There is definite evidence of the usage of nitrogenous material, but not of the usage of fat, even though the R.Q. may be very low. Under anaerobic conditions, however, the frog's heart resembles striped muscle in depending ultimately upon carbohydrate as the source of energy though the breakdown of phosphocreatine is probably the primary source [Clark, Eggleton and Eggleton, 1932]. Clark, Gaddie and Stewart [1932] have also shown that the continued power of the heart to contract in the absence of oxygen depends upon two factors: the presence of carbohydrate which may be broken down to lactic acid, and the supply of a sufficiently alkaline perfusion fluid to neutralize and remove the lactic acid formed. Thus a neutral perfusion fluid prevents the contraction from continuing for longer than a few minutes, since the lactic acid quickly accumulates in the heart. On the other hand, a fluid buffered to *p*H 8 with $NaHCO_3$ or Na_2HPO_4 allows the heart to contract, even without added glucose, for some hours at the expense of its own carbohydrate stores; for in this case the lactic acid is neutralized and removed by the perfusion fluid.

When the heart, perfused by alkaline Ringer fluid, has been exhausted anaerobically, it can be completely revived by the addition of glucose, an observation made by Freund and König [1927] and confirmed by Clark, Gaddie and Stewart [1932]. It is evident that the same restorative effect should be exerted by any substance which can replace glucose

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as an anaerobic source of energy, and by any substance which is an intermediate in the breakdown of carbohydrate to lactic acid, provided that its conversion to lactic acid liberates a sufficient amount of energy to allow of contraction. The frog's heart, exhausted by continued anaerobic contraction with an alkaline perfusion fluid, is thus an exceedingly convenient preparation for the study of carbohydrate metabolism in muscle. It is also, of course, suitable for investigating the action of various muscle poisons, of which iodoacetic acid is, at present, the most important.

Like skeletal muscle, cardiac muscle is prevented from contracting anaerobically by iodoacetic acid, which, however, does not abolish aerobic contraction [Clark, Eggleton and Eggleton, 1932].

The possibilities for cardiac muscle may therefore be summarized as follows:

A. Unexhausted heart contracting in oxygen, and oxidizing its own stores of carbohydrate and protein.

B. Exhausted heart contracting in oxygen, depleted of its stores of carbohydrate and available protein, benefited by addition of glucose and amino acids.

C. Unexhausted heart contracting in nitrogen, converting carbohydrate to lactic acid which must be removed from the cells by an alkaline perfusion fluid.

D. Exhausted heart contracting in nitrogen, depleted of carbohydrate, benefited only by sugar or intermediates in carbohydrate breakdown to lactic acid.

E. Unexhausted heart contracting in oxygen and poisoned by iodoacetic acid, probably using only amino acids.

F. Unexhausted heart contracting in nitrogen, poisoned by iodoacetic acid, probably dependent solely on phosphagen breakdown.

G. Exhausted heart contracting in oxygen, poisoned by iodoacetic acid, depleted of available nitrogenous material, probably benefited by amino acids and by lactic acid but not by carbohydrate.

The experiments described in this paper are concerned with conditions D and F; conditions E and G are being studied at present, and will form the subject of a further communication.

Methods.

Rana esc. (Hung.) was used. The perfusion of the isolated ventricle was carried out with a cannula in the auriculo-ventricular opening, and arranged so that isometric records could be taken when desired. The ventricle was stimulated electrically at any desired rate (see Appendix).

The apparatus was of similar type to that illustrated by Clark [1927, Fig. 1]. Air and nitrogen from a fine jet could be bubbled through the perfusion fluid, and the current of gas served to stir the fluid. The nitrogen was rendered oxygen-free by passing it over heated copper, and re-contamination was guarded against by closing the cannula with a rubber stopper fitted with a tube for the escape of the gas.

The Ringer fluid used had the following percentage composition: NaCl 0·65, KCl 0·015, $CaCl_2$ 0·012, $NaHCO_3$ 0·05. When gas was bubbled through this fluid the *p*H rose to about 8·5. Glucose and other substances in solution were introduced into the perfusion fluid through a hole in the rubber stopper, closed by a glass rod when not being used.

The sugars and glutathione used in our experiments were B.D.H. reagents. Methyl glyoxal was made by the method of Hoffmann and

Fig. 1. Typical recovery of an exhausted ventricle on addition of glucose. (Anaerobic.)

Neuberg [1930], dihydroxyacetone and glyceric aldehyde by the method of Hersant and Linnell [1931]. Iodoacetic and bromoacetic acids were synthesized from chloroacetic acid and precipitated from hot benzene solution by addition of petrol ether. The remaining iodo compounds were synthesized by Miss D. Mowat who kindly supplied us with samples.

Results and discussion.

(*a*) *The effect of glucose.* Fig. 1 is a typical record of the gradual exhaustion of the ventricle perfused by alkaline Ringer in an atmosphere of nitrogen and of the recovery on addition of glucose. For about an hour the heart contracts in nitrogen as strongly as in oxygen, but thereafter the contractions gradually lose strength until they finally disappear. The time required for this total exhaustion varies from as little as 2 to as much as 6 hours, according to the amount of glycogen available, the rate of stimulation, and the frequency with which the perfusion fluid is changed.

The importance of the first two of these factors is obvious. With regard to the third, it seems that the perfusion fluid washes out a part of some substance necessary for proper contraction, so that changing the perfusion fluid two or three times definitely hastens the exhaustion. More frequent changing, however, has no further effect. The nature of this substance will be considered further in section (*c*).

The recovery on addition of glucose is rapid, but is more so when, as in Fig. 1, the ventricle has not been allowed to become completely exhausted. Evidently, if the heart has completely stopped the diffusion of the sugar through the narrow cannula is relatively slow. When recovery is complete, *i.e.* in about 10 min. after addition of glucose, changing the perfusion fluid leads to a rapid re-exhaustion of the ventricle which can then be recovered again by glucose. This process of alternate exhaustion and recovery can be repeated many times on the same ventricle. Advantage was taken of this in controlling the experiments described later in this paper, for a trial of any substance was always sandwiched between two successful glucose recoveries.

(*b*) *Other carbohydrates.* Although it is known that the intact animal is capable of utilizing a variety of carbohydrates, it is not certain whether some, or all, of the tissues are able to use them directly, or whether they must first be converted to glycogen in the liver. It would seem *a priori* that glucose, fructose and mannose might all be used directly by muscle, since they possess a common enol form. However, Maclean and Smedley [1912] found that the hearts of the dog and rabbit, in presence of oxygen, utilized glucose and mannose fairly rapidly, and galactose to a slight extent, but maltose, fructose and xylose, not at all. The negative result with fructose was also obtained by Steinberg [1927] using rabbit's heart muscle, and by Ashford [1933] with brain. Working with embryonic tissues under anaerobic conditions, Dickens and Greville [1932] found that some were able to convert only glucose to lactic acid, while others were able to convert fructose as well.

In the isolated frog's ventricle the results with fructose were completely negative, whereas mannose restored the power of contraction as rapidly and completely as did glucose itself (Fig. 2). The other carbohydrates tested all gave negative results—*pentoses*: arabinose, ribose, xylose; *hexoses*: galactose; *disaccharides*: maltose, lactose, sucrose; *polysaccharides*: glycogen, starch.

Although Freund and König reported recovery after addition of glycogen to the exhausted heart, we have never succeeded in obtaining any trace of recovery. The size of the glycogen molecule makes its diffusion

into the cell somewhat unlikely, and of course such a diffusion is an essential preliminary to recovery. We have found, however, that minute traces of glucose are capable of bringing about some degree of recovery, and it is just possible that Freund and König used a specimen of glycogen slightly contaminated with glucose.

Fig. 2. The action of mannose on an exhausted ventricle (in absence of oxygen) compared with the action of glucose on the same ventricle.

(c) *Non-carbohydrate substances.* Since amino acids are capable of acting as sources of energy under aerobic conditions [Clark, Gaddie and Stewart, 1931], and in view of the statement by Freund and König that glycine and alanine restored the activity of the exhausted anaerobic heart, these substances were tested in the isolated ventricle exhausted in nitrogen. The results, however, were completely negative. Glycine and alanine were also found to be without effect on the strength of the

Fig. 3. Failure of alanine and glycine to cause recovery of the exhausted ventricle in nitrogen. Glucose restores power of contraction.

contraction of the unexhausted heart whether working in air or nitrogen (Fig. 3).

Clark, Gaddie and Stewart [1931] found that the addition of soaps to the perfusion fluid caused a rise in oxygen consumption by the isolated frog's heart without any change in the R.Q. The effect of soaps on the

exhausted heart was somewhat similar. Fig. 4 shows that the addition of 0·1 p.c. sodium oleate to the exhausted heart produced a temporary recovery. Sodium linoleate produced a similar recovery. It is to be noted, however, that this recovery with soaps could be made once only by each heart, and could not be repeated many times as in the case of recoveries with glucose. Evidently the fatty acid, unlike glucose, is not acting as a source of energy. This result agrees with Clark's [1913] hypothesis that soap restores some constituent of the cell which is removed by perfusion.

(*d*) *Possible intermediates in the breakdown of carbohydrate.* The conversion of carbohydrate to lactic acid most probably involves the splitting of the hexose molecule into two triose molecules. As a preliminary stage, it seems that the hexose is first esterified with phosphoric acid, and there

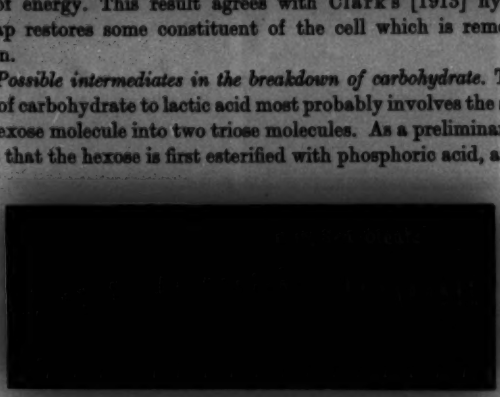

Fig. 4. Slight recovery on addition of sodium oleate to the fluid perfusing the ventricle partly exhausted in nitrogen.

is evidence that the triose molecules are also produced in the form of phosphoric esters. In this connection of course the substances to be considered are glyceric aldehyde and dihydroxyacetone.

Trial of these two trioses on the exhausted ventricle, however, gave very disappointing results. Dihydroxyacetone was without effect; glyceric aldehyde did appear to increase the force of contraction, but the response to stimulation became very irregular and ceased altogether within a few minutes. Larger concentrations of glyceric aldehyde were definitely toxic, at once preventing the ventricle from responding to stimulation. The heart recovered, however, if the glyceric aldehyde was washed out and replaced by glucose. Glucose gave no recovery unless the glyceric aldehyde was first removed (Fig. 5).

It may be objected that the true intermediates in carbohydrate breakdown are the phosphoric esters of these trioses and not the trioses themselves. It is arguable, however, that if the heart muscle is able to

use glucose directly, *i.e.* to esterify it, it should be able to do the same with the trioses. This is not necessarily the case, since the esterified state of the trioses may be due merely to their production from a hexose diphosphate. It must be admitted, in fact, that further work is necessary on this point. Our results so far as they go, however, suggest that while minute amounts of glyceric aldehyde may be utilized by the heart muscle, larger concentrations are definitely toxic.

There remains the possibility that the trioses prepared synthetically exist in inactive forms very different from these produced and utilized in the enzymatic breakdown of hexose. This point need not be stressed, for the possibilities of keto-enol changes in these compounds are well known, but it is obvious that in face of these objections our negative results cannot

Fig. 5. The action of glyceric aldehyde on the exhausted ventricle in nitrogen, increase in the force of contraction, but irregularity in the response to stimulation.

be taken as eliminating these two compounds from consideration as intermediates in lactic acid formation.

Whatever triose or trioses may be produced from hexose, their further breakdown most probably furnishes either methyl glyoxal or pyruvic acid. The former could of course be produced from either dihydroxyacetone or glyceric aldehyde by intra-molecular change, the simultaneous oxidation of one carbon atom and reduction of another within the same molecule, the net result being the elimination of water. Pyruvic acid formation involves more than this, the addition of an extra atom of oxygen. Consequently, since the reaction, if it takes place at all, does so anaerobically, a second molecule of some kind must be reduced. In other words, there is the probability of the simultaneous formation of glycerol (or glycerophosphoric acid).

Methyl glyoxal and sodium pyruvate were tested on the exhausted ventricle; the former produced a rapid and sustained recovery, the latter

was without effect (Fig. 6). It may be noted incidentally that the recovery in presence of methyl glyoxal was less complete than that produced by glucose, and that, after a partial recovery was produced by the addition of methyl glyoxal, a further recovery was produced by glucose.

This result lends support to the view that methyl glyoxal is an intermediate in the formation of lactic acid from carbohydrate. It must be borne in mind, however, that glyoxalase may conceivably not form a part of the enzyme system concerned in carbohydrate utilization. In their essentials, the experimental results merely indicate that the energy liberated in the conversion of methyl glyoxal to lactic acid is adequate for contraction and that the muscle can use it for that purpose. We know that aerobically the muscle is capable of utilizing energy from a variety of sources. It may well be that the same is true under anaerobic conditions, but that ordinarily the breakdown of sugar is the only one available, and that the supply of methyl glyoxal allows an utterly abnormal mechanism to function. We may say therefore that the possibility of the utilization of methyl glyoxal is proved but that the inclusion of methyl glyoxal as an intermediate in carbohydrate breakdown remains unproved. Further consideration of this matter is best deferred until a later section of the paper.

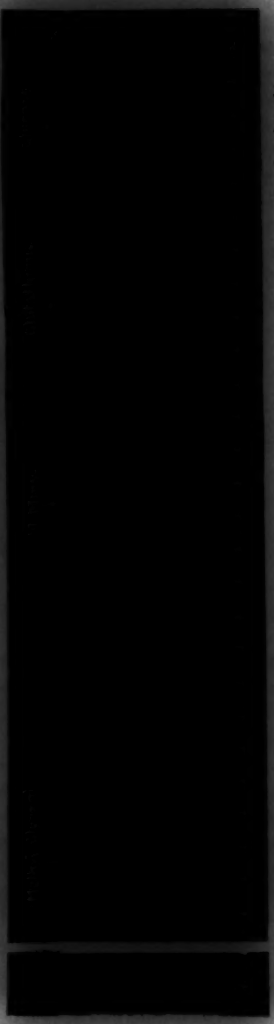

Fig. 6. The effect of methyl glyoxal on the exhausted ventricle in nitrogen, partial recovery, increased somewhat by glutathione, and converted to full recovery by glucose.

The conversion of methyl glyoxal to lactic acid involves the addition of a molecule of water, the reduction of the keto group and the oxidation of the aldehyde group. Thus no other substance (except water) is involved. Pyruvic acid on the other hand is reduced to lactic acid so that under anaerobic conditions some other molecule must be oxidized. In the formation of pyruvic acid glycerol is probably produced, and since there is no evidence of its accumulation in the muscle, it must be supposed to be the hydrogen donator in the reduction of pyruvic acid. Consequently the fact that pyruvic acid alone is unable to restore the activity of the ex-

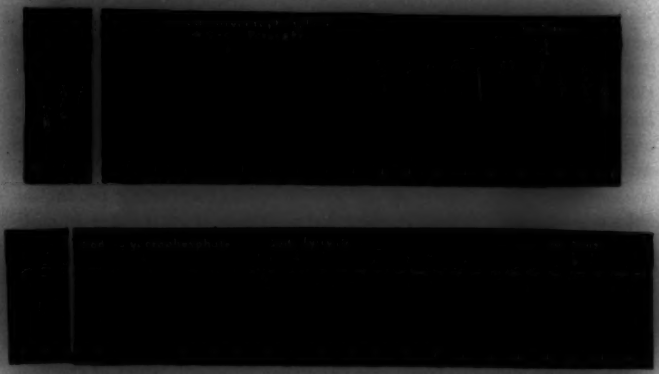

Fig. 7. Partial recovery of the ventricle exhausted in nitrogen by means of equimolecular mixture of sodium pyruvate and sodium glycerophosphate. The upper tracing shows the most complete recovery obtained, the lower shows the usual response. Note that sodium glycerophosphate alone is without effect. Sodium pyruvate alone (not shown) is equally ineffective.

hausted ventricle must be regarded as inconclusive in eliminating pyruvic acid as an intermediate in lactic acid formation. We therefore tested an equimolecular mixture of sodium pyruvate and sodium glycerophosphate. As Fig. 7 shows a partial recovery was produced, the effect never being as great as that of methyl glyoxal and sometimes little more than bare maintenance of weak contractions. Here then we have definite evidence in favour of the chain of reactions which involve the production of pyruvic acid and glycerol—the chain postulated by Embden and by Meyerhof for the breakdown of carbohydrate in skeletal muscle extracts and in yeast fermentation.

In order to gain further evidence as to the power of the heart muscle to form phosphoric esters other than those of hexose, we further tested equimolecular mixtures of sodium pyruvate and glycerol, which gave no restoration of the power to contract.

A number of other substances which have been suggested as possible intermediates in lactic acid formation were tested, but with uniformly negative results: succinic acid, hydroxybutyric acid, aceto-acetic acid, fumaric acid, maleic acid, acetic acid, acetaldehyde, ethyl alcohol and ethylene glycol.

It is especially noteworthy in the experiments with glycerophosphoric acid and pyruvic acid (as with methyl glyoxal) that after recovery had proceeded to its maximum, a still further increase in the strength of contraction was produced by the addition of glucose. If it be accepted that glycerophosphoric acid and pyruvic acid together form one stage in lactic acid production it is justifiable to conclude further that with the utterly abnormal amounts present the enzymes concerned in the reaction must have been completely saturated. Moreover, their presence in such concentration would probably suffice to prevent their further production from added sugar. It follows then that if glucose is to produce an additional effect, it must do so by being broken down in some other way than that involving the production of these two substances. The alternative though (in our opinion) less probable explanation is that the energy liberation in the reaction: glycerophosphoric acid + pyruvic acid = lactic acid + triose phosphate, accounts for a fairly small part of the total energy of the change glucose → lactic acid; and that glucose in spite of the high concentration of glycerophosphoric and pyruvic acids is still converted to these two substances with liberation of more available energy.

A similar argument, of course, applies to the experiments with methyl glyoxal, so that both series suggest the existence of two routes for the conversion of carbohydrate to lactic acid.

(*c*) *The action of iodoacetic acid and related compounds.* Under aerobic conditions low concentrations of sodium iodoacetate (*i.e.* conc. of about 1 : 20,000 in the perfusing fluid) have little or no effect for several hours, but when oxygen is excluded, the heart ceases to contract after about 7 min. (Fig. 8). Re-admission of oxygen to the paralysed heart (together with massage if contraction has entirely ceased) causes a complete revival, and this process of alternate stoppage by iodoacetic acid in absence of oxygen, and revival by oxygen can be repeated many times. The concentration of sodium iodoacetate necessary to stop the heart in absence of oxygen is about 0·005 p.c. or 0·25 millimol. Lower concentrations than

this are either without action or diminish the power of anaerobic contraction gradually without completely abolishing it (except perhaps after so long that the stoppage may be due as much to lack of carbohydrate as to the poisonous effect of the iodoacetic acid). Concentrations higher than 0·005 p.c. do not increase the rapidity of the action, but high concentrations are rapidly toxic even in the presence of oxygen.

Sodium monobromoacetate acts as rapidly as sodium iodoacetate and in equal concentrations; sodium monochloroacetate, however, is without effect, even in concentrations of 2·5 millimol. This finding is of importance in view of (*a*) the observation of Dudley [1931] that iodoacetic acid inhibits glyoxalase, (*b*) the results of Lohmann [1932] indicating that

Fig. 8. The action of iodoacetic acid on the fresh ventricle under anaerobic conditions. In this case the heart was allowed to stop completely before oxygen was admitted, and it was necessary to re-start contraction mechanically.

iodoacetic acid inactivated the co-enzyme of glyoxalase and of Jowett and Quastel [1933] indicating that this co-enzyme is glutathione; (*c*) the finding by Dickens [1933 *a*] that *in vitro* iodoacetic acid reacts with glutathione, liberating hydriodic acid.

Since it has been found [Dickens, 1933 *b*] that glutathione reacts, in slightly alkaline solution, with all three mono-halogen derivatives of acetic acid, liberating the halide acid, the results on the frog's ventricle suggest that iodo- and bromoacetic acids have some further action in addition to their inactivation of co-glyoxalase. Monochloroacetic acid reacts with gluthathione at only about 1/10 the velocity with which monobromoacetic acid reacts. Nevertheless, it is able, in blood, to cause an appreciable slowing of the rate of disappearance of glucose, in a concentration about three times as great as the effective concentration of bromo- and iodoacetic acids [Mowat and Stewart, 1934].

Mono-iodo propionic acids, both α and β isomers, are without action on the frog's ventricle even in high concentrations. Iodoethyl alcohol, $CH_2I.CH_2OH$, is also unable to prevent contraction under anaerobic conditions. These facts are peculiar since all three substances have been found to have an appreciable effect in inhibiting the conversion of glucose to lactic acid in blood [Mowat and Stewart, 1934].

(*f*) *The effect of glutathione on the ventricle poisoned by iodoacetic acid.* Since methyl glyoxal was found to restore activity to the ventricle exhausted in absence of oxygen, and since strong evidence had been produced by Lohmann, Jowett and Quastel, Dickens, etc., that iodoacetic acid reacted with and destroyed glutathione, thereby preventing the conversion of methyl glyoxal to lactic acid, we attempted to

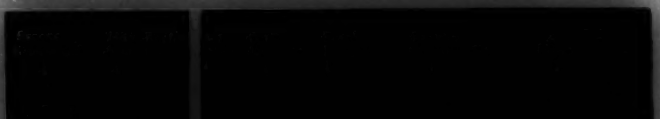

Fig. 9. Ventricle in nitrogen. Addition of iodoacetic acid to fluid already containing glutathione reduced the contraction only very slowly, so slowly that the effect was due largely to exhaustion of carbohydrate stores. Addition of glucose at this stage further reduced the force of contraction. Removal of glucose by washing and addition of more glutathione brought about slow recovery.

restore activity to the ventricle paralysed by iodoacetic acid, by addition of an excess of reduced glutathione to the perfusion fluid.

In our first experiment glutathione (neutralized) was added to the perfusion fluid of a heart contracting anaerobically to make a 1·25 millimol. solution. There was no effect on the strength of contraction. Sodium iodoacetate was then added in the usual concentration (0·25 millimolar) and at the end of 15 min. the heart was still contracting with almost undiminished strength. A second addition of iodoacetate equal to the first was still insufficient to cause paralysis (Fig. 9).

This experiment, however, was really unsatisfactory, since the *p*H of the fluid was that at which glutathione and iodoacetic acid rapidly interact *in vitro*, so that the glutathione in the perfusion fluid was probably acting merely by preventing the iodoacetate from ever reaching the muscle cells. A second experiment was therefore made. Sodium iodo-

acetate was added in the usual amount, and, when the ventricle had almost ceased to contract, the perfusion fluid was removed and replaced by fresh Ringer's solution containing glutathione. The ventricle at once began to contract, and the strength of contraction gradually increased to about half the normal, at which it remained for over an hour, when the experiment was stopped. The observation was of course repeated several times, and it was shown that mere changing of the perfusion fluid without supplying glutathione was quite unable to restore activity to the ventricle. In fact repeated change of the perfusion fluid, the heart being meanwhile kept contracting in oxygen, was quite incapable of removing the effect of iodoacetic acid, and exclusion of oxygen at any time resulted in the rapid cessation of contraction (Fig. 10).

In this second type of experiment the glutathione is evidently reversing the effect of the iodoacetic acid by some action within the muscle itself. This action may and probably does consist in restoring the muscle glutathione previously destroyed by the iodoacetic acid. The recovery after addition of glutathione is never complete, however, and this fact, taken in conjunction with others recorded in the preceding pages, suggests that iodoacetic acid has other effects than that of inactivating co-glyoxalase and that these other effects are irreversible by any means yet discovered.

Cysteine, it may be noted, is capable of preventing the action of iodoacetic acid subsequently added to the perfusion fluid, but is quite unable to revive the ventricle already poisoned by iodoacetic acid.

It is remarkable that if glucose be added to the perfusion fluid of a heart poisoned by iodoacetic acid and revived by glutathione, the heart rapidly ceases to contract. (The simultaneous presence of glutathione and glucose has no effect on the unpoisoned heart.) The poisonous effect of glucose under these conditions is removed by merely changing the perfusion fluid, *i.e.* removing the glucose (Fig. 10).

As an explanation of this phenomenon it may be suggested that one (at least) of the intermediates in the conversion of glucose to lactic acid is toxic except in minute concentrations (cf. the experiments with glyceric aldehyde), and that iodoacetic acid affects the mechanism of lactic acid formation from this intermediate. Recovery by glutathione may be supposed sufficient to allow the removal of the toxic substance as quickly as it is formed from the normal sugar reserves of the heart. Addition of what is in effect an enormous amount of readily available sugar then causes the accumulation of the toxic substance at a rate much greater than that at which it can be removed. It may be recalled here, however,

Fig. 10. Ventricle in nitrogen, poisoned by iodoacetic acid, restored by addition of glutathione and change of perfusion fluid, and again poisoned by addition of glucose.

Fig. 11. Ventricle in nitrogen, slowly poisoned by sodium arsenite, restored by glutathione and change of perfusion fluid, and, after slow exhaustion (of carbohydrate stores), restored by glucose.

that glucose was incapable of restoring activity to the exhausted heart in the presence of glyceric aldehyde.

(*g*) *The action of arsenic.* Voegtlin and his collaborators [1923, 1931] have adduced evidence in favour of their hypothesis that trivalent arsenic combines with reduced glutathione and that the latter has therefore a protective action against arsenic in, *e.g.*, rats, rabbits, trypanosomes, etc.

If the two substances do combine, it follows that small quantities of arsenic should exert an effect on muscle similar to that of iodoacetic acid. It should, in fact, prevent the anaerobic contraction of muscle if the contraction depends upon the activity of glyoxalase, provided it is not added in amounts too great to be dealt with by the glutathione present. Excessive amounts of arsenic, of course, would in all probability act as a general enzyme poison. Under the conditions we are imagining:

(1) the muscle should be capable of contracting when supplied with oxygen but not in absence of oxygen (showing that the enzymes concerned in non-carbohydrate oxidation or aerobic carbohydrate breakdown are intact);

(2) the power of anaerobic contraction should be restored by reduced glutathione.

We find (Fig. 11) that sodium arsenite, at a concentration of 1/50,000 in the perfusion fluid, gradually abolishes the power of contraction in the isolated ventricle stimulated under anaerobic contraction. The action is much slower than that of iodoacetic acid, but is much more rapid than that of mere exhaustion of the carbohydrate reserves. Admission of oxygen restores the power of contraction to the ventricle poisoned in this way, and the power is again lost if the oxygen is excluded.

When anaerobic conditions are maintained, the poisoned ventricle is not revived by washing with normal Ringer nor by the addition of glucose. (In much higher concentrations arsenic prevents the heart from contracting even in oxygen—*i.e.* it acts as a general enzyme poison. Under these conditions, its effect on aerobic contraction can be removed by repeated washing.) It is, however, revived by glutathione (best after change of perfusion fluid to remove excess of arsenic) and the power of contraction is then retained until the carbohydrate stores are exhausted in the normal way, when glucose brings about a further recovery (Fig. 11).

The heart poisoned by this low concentration of arsenic and restored by glutathione appears to be quite normal. Changing the perfusion fluid several times, so as to remove circulating glutathione, does not decrease

the strength of contraction, so that the glutathione supplied must have penetrated the tissue and become fixed in the same way as the normal glutathione of the muscle.

It is evident that under the experimental conditions the main action of arsenic consists in its interaction with glutathione. It is not a general enzyme poison, and since glucose produces its normal effect after recovery of the poisoned heart by glutathione and exhaustion of carbohydrate stores, there is no sign of an effect on any other factor concerned in glucose breakdown, *i.e.* no accumulation of toxic intermediates. Since glutathione is known to be the co-enzyme of glyoxalase, it follows that methyl glyoxal is an intermediate in the formation of lactic acid from glucose in cardiac muscle.

It seems probable indeed that in cardiac muscle this is the main route available for the anaerobic breakdown of glucose, since the production of lactic acid *via* triose phosphates and pyruvic acid is stated [Meyerhof, 1933] to be independent of glutathione. It is possible that the pyruvic acid route is primarily that taken by glycogen, since in the experiments recorded here the glycogen reserve was already depleted at the time recovery with glutathione took place, as evidenced by (*a*) the partial nature of the recovery, and (*b*) the relatively short time before addition of glucose became necessary.

Moreover, since the production of methyl glyoxal is probably not interfered with, it follows that either a very low concentration of methyl glyoxal stops further breakdown of carbohydrate, or the energy liberated in the conversion of glucose to methyl glyoxal is very small.

(*h*) *Discussion of results.* It seems highly probable that there are at least two mechanisms for the breakdown of carbohydrate to lactic acid in living tissues. On the one hand there is a considerable body of evidence in favour of the supposition that methyl glyoxal is formed as an intermediate stage, and on the other hand much experimental work indicates that pyruvic acid occupies a similar position.

Methyl glyoxal is of course rapidly converted to lactic acid by a great many tissues since the enzyme concerned—glyoxalase—is very common. This, however, is not to be regarded as strong evidence in favour of the production of methyl glyoxal from sugar. Much more important in this respect are the demonstration that methyl glyoxal can be utilized in place of sugar by the exhausted heart, the recovery in presence of glutathione of the heart poisoned by iodoacetic acid or by arsenic, and the isolation of methyl glyoxal from muscle pulp under various conditions. Thus Case and Cook [1931] detected small amounts of the substance in muscle pulp

under both aerobic and anaerobic conditions; Toeniessen and Fischer [1926], Arayama [1928], Sym [1931], Inoue [1931], Barrenscheen *et al.* [1931, 1933], and others have all demonstrated the power of muscle pulp to produce methyl glyoxal (often in good yield) from hexose diphosphate. In the presence of muscle pulp, pancreatic extract, and iodoacetic acid, Barrenscheen, Braun and Dreguss found that glycogen also gave rise to methyl glyoxal.

Pyruvic acid has also been detected in muscle pulp under the same conditions as methyl glyoxal [*e.g.* Case and Cook, 1931]; Meyerhof [1933] has shown that muscle contains enzymes capable of producing lactic acid from a mixture of pyruvic acid and glycerophosphoric acid, and that pyruvic acid is produced during the breakdown of sugar in muscle extract; and we have found that these two substances are together capable of restoring some power of contraction to the frog's ventricle exhausted anaerobically.

It seems to be established therefore that both of these substances are formed during the production of lactic acid from carbohydrate. It follows either that the one must be converted into the other or that there are two ways in which lactic acid may be formed from carbohydrate.

The chain of reactions, methyl glyoxal→pyruvic acid→lactic acid, is conceivable, although Jowett and Quastel [1933] have adduced evidence to support the supposition of direct addition of water to an addition compound of methyl glyoxal and glutathione. Case considers that methyl glyoxal is converted to pyruvic acid and that this is the source of pyruvic acid in muscle glycolysis. His evidence for this, however, is of a purely negative character, in that he has failed to detect any other source of the observed pyruvic acid. Nevertheless, he failed to obtain pyruvic acid from added methyl glyoxal and was driven to postulate an active form of the latter in spite of the fact that synthetic methyl glyoxal readily gives rise to lactic acid.

It is worth mentioning, too, that Neuberg and Kobel [1930] described the production of pyruvic acid from hexose diphosphate by yeast as occurring later than that of methyl glyoxal. It is obvious, therefore, that the possibility of methyl glyoxal and pyruvic acid lying on the same line of breakdown must be considered, though it can be definitely accepted only after more positive evidence has accumulated. On this view, however, it becomes difficult to explain Meyerhof's statement that dialysed muscle, in presence of added adenosinetriphosphoric acid and magnesium, produces lactic acid from carbohydrate—*i.e.* under conditions in which glyoxalase cannot act.

The second possibility—that there are two separate enzyme systems each converting carbohydrate to lactic acid in its own way—is supported by much evidence and explains many phenomena which would otherwise be obscure. It is to be noted that the two systems would not necessarily act on the same carbohydrate and that they might well occur in varying proportions in different tissues.

Moreover, they might assume different relative degrees of importance in the same tissue at different times or under different conditions.

Recently Meyerhof [1933] has advanced strong evidence in favour of the view that carbohydrate is converted *via* hexose diphosphate to triose phosphates, thence to pyruvic and glycerophosphoric acids, and so to lactic acid. In so doing he refuses to admit the possibility of methyl glyoxal having any share in the reaction, basing his objection chiefly on the following facts:

(*a*) Glutathione is essential for the conversion of methyl glyoxal to lactic acid.

(*b*) Lactic acid is produced from glucose even by dialysed muscle extracts, after the addition of adenosine—triphosphoric acid and magnesium.

He ignores, however, the considerable body of positive evidence in favour of methyl glyoxal, and completely overlooks the possibility of a double mechanism. Yet the view suggested in the present paper is directly supported, *inter alia*, by the work of Ashford on brain tissue [1933]. Ashford found that glycogen, hexose diphosphate, hexose monophosphate, glucose and mannose are all converted to lactic acid by brain tissue, as also are methyl glyoxal (more rapidly than glucose) and pyruvic acid (less rapidly than methyl glyoxal). If, however, any one of the group: glycogen, hexose diphosphate and hexose monophosphate, was incubated with brain tissue along with glucose or mannose, the lactic acid produced was the sum of the amounts obtained from the two substances separately (when lactic acid formation was proceeding at the maximal rate). Ashford draws the obvious deduction that there must exist two enzyme systems, one dealing with glucose or mannose, the other with glycogen or hexose phosphate.

The evidence from our own work in support of this view may be summarized as follows:

(1) Recovery of the exhausted heart by methyl glyoxal or by pyruvic acid (+glycerophosphoric acid) is incomplete.

(2) After maximal recovery by these substances, glucose brings about a further recovery.

The recovery of the ventricle on addition of the substances suspected of being lactic acid precursors is evidence that they can be utilized. The fact that the recovery is only partial might be considered to indicate that the heart was in an abnormal or damaged condition, were it not for the further observation that after their removal, glucose invariably brought about a complete recovery. The quantities of the intermediates added to the perfusion fluid were obviously greatly in excess of any amounts which might be formed normally during the breakdown of carbohydrate, and it is reasonable to suppose that they were sufficient to saturate the enzymes concerned. Consequently the additional recovery given by glucose added in presence of intermediates is strong evidence of the existence of alternative routes.

It might be objected that the extra recovery due to glucose is attributable to energy liberated by the breakdown of glucose to methyl glyoxal or pyruvic acid as the case may be. Unfortunately, the published values for the heat of combustion of methyl glyoxal differ too widely for calculation of the energy released during the earlier stages to be of any value. On the whole, the indications are that this energy must be very small. In the case of pyruvic acid, the position is complicated by the fact that some other compound must be simultaneously oxidized, and again, one can do no more than speculate on the magnitude of the energy changes involved.

(3) Monochloroacetic acid, α- and β-iodopropionic acids, and iodoethyl alcohol are without action on the isolated frog's ventricle contracting anaerobically. They are, however, capable of inhibiting glycolysis in blood [Mowat and Stewart, 1934]. This observation of course indicates a difference between the relative importance of the two hypothetical systems in cardiac muscle and in blood.

(4) Even in large excess, glutathione is unable to restore full power of contraction to the ventricle poisoned by iodoacetic acid. Yet it completely abolishes the power of iodoacetic acid to inhibit glycolysis in blood [Mowat and Stewart, 1934].

(5) Glutathione when added to the fluid perfusing a frog's ventricle which had been poisoned in the absence of oxygen with the minimum amount of trivalent arsenic, completely restored the power of anaerobic contraction. It is important to notice, however, that the amount of arsenic added was too small to produce a general enzyme poisoning as was shown by the power of the poisoned ventricle to contract normally in oxygen. In other words the arsenic, like iodoacetic acid, had specifically inhibited the enzymes responsible for the breakdown of carbohydrate to lactic acid.

Even the two-route hypothesis is insufficient to explain the fact that the ventricle which has been poisoned and recovered by glutathione behaves differently towards glucose according to whether the poison used was iodoacetic acid or trivalent arsenic. The toxic action of glucose upon a ventricle poisoned by iodoacetic acid (and recovered by glutathione) can be explained by postulating the formation of a toxic intermediate. No such toxic intermediate is formed, however, in the case of arsenic. Since, however, arsenic stops all lactic acid formation (the ventricle, so poisoned, is not recovered by glucose alone), it follows that arsenic has a double action. It inhibits glyoxalase by combining with glutathione just as does iodoacetic acid; it differs from iodoacetic acid, however, in inhibiting the primary stages of carbohydrate breakdown at an earlier point. The toxic intermediate, on the basis of Meyerhof's explanation of the action of iodoacetic acid and our own findings with respect to dihydroxyacetone and glyceric aldehyde, may possibly be phosphoglyceric acid, which we have not yet had the opportunity of testing on the frog's ventricle.

It is not suggested that the evidence presented in this paper is sufficient to prove the existence of a double line for the breakdown of carbohydrate to lactic acid. It is, however, suggested that this hypothesis is to some extent supported by evidence and that it offers the best explanation, at the moment, of a number of puzzling phenomena.

Summary.

1. The isolated ventricle of the frog, exhausted of carbohydrate by continued contraction in the absence of oxygen, is revived by the addition to the perfusion fluid of glucose or mannose, but not fructose, galactose, arabinose, ribose, xylose, maltose, lactose, sucrose, glycogen, starch, glycine, or alanine.

2. Under similar circumstances, sodium oleate and sodium linoleate produced temporary recovery which, unlike that caused by glucose and mannose, could not be repeated after re-exhaustion of the same ventricle.

3. Under similar circumstances, methyl glyoxal produced a partial recovery, convertible to full recovery by the addition of glucose; pyruvic acid, sodium glycerophosphate, and dihydroxyacetone were separately without effect; pyruvic acid and sodium glycerophosphate together produced slight recovery, increased by glucose to full recovery; and dl-glyceric aldehyde increased the force of contraction temporarily, but exerted a toxic effect.

4. The isolated ventricle, contracting in absence of oxygen, was rapidly stopped by iodo- and bromoacetic acids in concentrations of about 0·25 millimol.; monochloroacetic, α- and β-iodopropionic acids and iodoethyl alcohol were without effect even in much higher concentrations. The effect of iodoacetic acid was not removed by frequent changing of the perfusion fluid.

5. The isolated ventricle, poisoned by iodoacetic acid in the absence of oxygen, was restored to activity by addition to the perfusion fluid of excess of reduced glutathione. The recovery was not complete, and the addition of glucose rapidly abolished contraction. Cysteine was able to prevent the action of iodoacetic acid but not to restore activity to a ventricle which had been poisoned by iodoacetic acid.

6. Sodium arsenite, in a concentration of 0·25 millimol., slowly abolished the power of contraction of the isolated ventricle in absence of oxygen but not in presence of oxygen. The ventricle, so poisoned, partially recovered the power of anaerobic contraction on the addition of reduced glutathione, and the further addition of glucose completely restored the power of contraction.

7. The bearing of these results on the mode of carbohydrate breakdown is discussed, and it is suggested that there may be two routes for the formation of lactic acid—one *via* methyl glyoxal, and the other *via* pyruvic acid.

We wish to acknowledge gratefully the interest which Prof. A. J. Clark has taken in the progress of this work. We have also to thank the Moray Research Fund of this University for a grant in aid of the expenses.

APPENDIX.

A modification of the magnetic tipper previously described [Condon, 1913] was devised by Mr N. E. Condon to give electrical stimuli at regular intervals. The outer jacket of a Liebig condenser was arranged to give a constant flow of water (Fig. 12, *L*) into a bucket, *A*, so making and breaking at regular intervals an electric contact, *E*. The platinum contacts of the older apparatus were replaced by a piece of platinum wire fixed to the bucket arm and arranged to dip into a cup of mercury at *E*. This provides the make-and-break of the primary circuit, and the mercury switch has the advantage over the platinum contact of allowing a longer time for the passage of current through the primary coil.

The essential modification of the older apparatus consisted in a small vulcanite platform *V*, on which were mounted two metal cups *F* contain-

ing mercury, and a hinged rod *G* provided at one end with a means *H* of shorting the two cups and, at the other end, a small metal plate *P*. Through a 3/16 in. hole in this plate passed a curved piece of steel banjo wire of gauge 29 (K), with a slight kink near the end. The curvature of the wire was so arranged that when the bucket was empty, the wire just

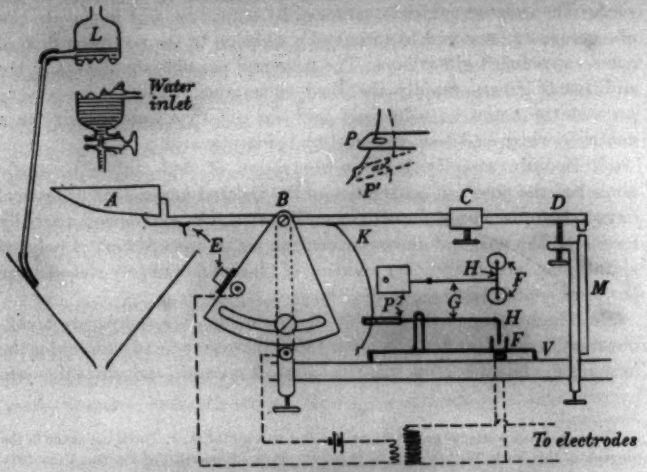

Fig. 12. Modification of Condon's magnetic tipper, designed to give electric stimuli at regular intervals.

touched the end of the hole remote from the hinge. When, however, the bucket was full and tipping, the wire engaged the opposite side of the hole. Thus for a short period during the return journey of the bucket, the wire depressed the metal plate and so lifted the wire which shorted the metal cups (which formed a switch in the secondary circuit).

The wiring is sufficiently shown in the diagram.

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GLYCOGEN STORAGE AND LÆVULOSE TOLERANCE.

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Carbohydrate tolerance tests, especially when done with lævulose, are thought to give some indication of liver function [Isaac, 1920; Isaac and Siegel, 1928; von Bergmann, 1932; Stroebe, 1932; Kimball, 1932, etc.]. Hofmeister [1890] was the first to point out that dogs who had been starved for a long time developed glycosuria after the ingestion of carbohydrates (hunger-diabetes). Barrenscheen [1914] showed by transfusion methods that less glycogen than normal was formed in a liver if it were from a starved animal and contained little glycogen. Bang [1913] demonstrated a high and prolonged blood-sugar curve following glucose ingestion in rabbits after a period of starvation. Staub [1922] found a similar type of blood-sugar curve after fasting in man. In view of the previous work of Barrenscheen, this type of blood-sugar curve was taken to mean that the liver cell could not form glycogen when it contained little itself [Lesser, 1920; Staub, 1922; Lichtwitz, 1930]. Isaac [1920] showed that after ingestion of lævulose the blood sugar in normal man rises only slightly, whereas with a damaged liver there is a considerable rise.

The small normal rise of blood sugar in man after ingestion of lævulose was believed to be due to unchanged lævulose [Isaac, 1920]. The micro-method of estimating lævulose introduced by van Crefeld [1927] showed, however, that it is always mainly due to glucose [van Crefeld and Ladenius, 1928; and others]. In the case of experimental damage to the liver (chloroform, hydrazine sulphate) and disease in man the larger rise is partly due to unchanged lævulose but much more to glucose, in some cases indeed to it alone; Corley, 1929; Heinecke and Peters, 1930; Steinitz, 1932; and others]. The unchanged lævulose when found is in all probability due to the failure of such a liver to convert lævulose fully into glucose, a conversion which was shown by Mann and Magath [1921] to be a function of the normal liver. The increase in

glucose was assumed, on the basis of Barrenscheen's experiments, to be due to the inability of a liver containing too little glycogen to make any more. Vogt [1932], under Stroebe, held the definite view that "only a liver rich in glycogen can deal adequately with large quantities of carbohydrate. A raised and prolonged blood-sugar curve after lævulose justifies the conclusion that the liver contains little glycogen." Buettner and Neuhaus [1931], Stroebe [1932], Steinitz [1932] and others consider this type of blood-sugar curve as evidence of the inability of the damaged liver to form or store glycogen. There is no experimental evidence to justify these opinions.

The following paper, therefore, deals with the problem whether a lack of glycogen in the liver cells impairs the power of forming glycogen after ingestion of lævulose and whether the blood-sugar curve is determined by the readiness with which glycogen can be formed or stored in the liver.

Methods.

Groups of male rats were submitted to different periods of starvation. The blood sugar and the average resting glycogen content of the liver and muscles were determined, and then blood sugar, liver and muscle glycogen estimated at intervals after the administration of a standard dose of lævulose.

Each series consisted of nine or ten animals of about 150 g. body weight which were kept under the same conditions two weeks before the experiment. Some (3 to 5) were then killed, while the rest received the sugar and were killed one at the end of each hour up to the sixth hour after it. The standard meal consisted of about 500 mg. of lævulose which was dissolved in 1 c.c. of water and introduced into the stomach by a fully filled 1 c.c. syringe fixed to the end of a stomach tube. At the end of the experiment the syringe was again fully filled with the lævulose solution employed in the experiment, then emptied into a 200 c.c. flask, made up with water and the exact amount of lævulose estimated in an aliquot part by Hagedorn-Jensen's method. Immediately after the death of the rat the liver and the skinned left leg were frozen in liquid air, and the glycogen content estimated by Pflüger's method. The muscular tissue remained almost perfectly intact by removing the leg from the pelvis. Blood was obtained from the heart with filter paper or by bleeding into a dish containing oxalate crystals. Estimation of blood sugar was made by Hagedorn-Jensen's method. The amount of lævulose absorbed was calculated by subtraction of the sugar still found in the alimentary tract after death from the total amount administered.

In order to determine the lævulose content of the gut, the whole alimentary tract was removed between ligatures and cut in pieces. The fluid was expressed from the segments which were then washed and all the fluid so obtained was filtered through glass wool. The sugar content of an aliquot part of the filtrate was then determined. No appreciable sugar content was found by this method in the empty alimentary tract.

Results.

Lævulose absorption from the gut.

The amount of sugar absorbed has been stated by Cori [1925, 1926] to depend very much on the length of the previous fasting period. He found that 77 mg. of lævulose were absorbed per hour per 100 g. rat with a previous fasting period of 48 hours, whereas with only 24 hours more than 100 mg. were absorbed. In the present experiments (Table I) no marked difference in absorption was found in rats starving for 12, 24, 48 or 96 hours. It was, however, considerably faster in winter, average 200 mg. in the first hour, than it was in the summer, when it averaged 131 mg. The average for all rats examined was found to be 174 mg. in the first hour, 142 mg. in the second and 125 mg. in the third. Absorption was complete at the end of the third hour. The values of blood sugar were not affected by the rate of absorption.

Table I. Lævulose absorption from the alimentary tract.

	Rate of absorption (in mg. per 100 g. rat)		
	1st hour	2nd hour	3rd hour
Rats starving 12 hours	180	148	128
„ „ 24 „	156	130	—
„ „ 48 „	149	135	115
„ „ 96 „	164	142	123
Rats killed during winter	202	157	127
„ „ „ summer	131	119	114
Average of all rats	174	142	125

Glycogen formation in the wall of the intestine.

If glycogen were formed in the intestinal wall during the absorption, the subsequent breakdown of this glycogen might contribute to the raising of the blood sugar. Accordingly estimations of glycogen in the intestinal wall were made; the amounts found were negligible and showed little or no change during and after lævulose absorption.

The blood sugar.

In this and the following sections the results are best set out by grouping the experiments thus.

Group I consisted of well-fed rats without a long previous fasting period. They were fed with 1 g. lævulose per 150 g. body weight on the eve of the experiment (12 hours before it) and then kept starving. In this group 52 rats were employed, 25 of them being killed at different intervals after the lævulose meal. In these latter the average increase of blood sugar was 7 mg. per 100 c.c.; in 68 p.c. of them not more than 10, in 32 p.c. between 10 and 50 mg. per 100 c.c.

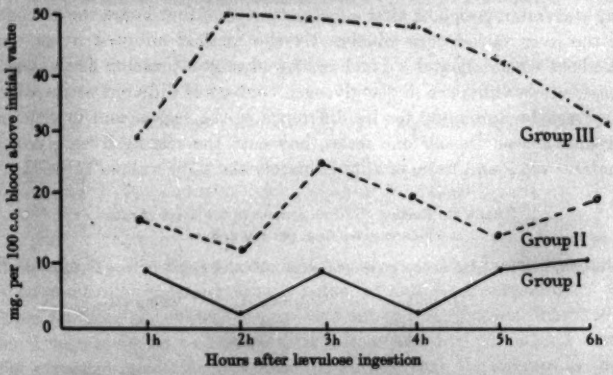

Fig. 1. Blood-sugar increase after lævulose meal. Figures given in mg. per 100 c.c. above initial level. Group I: well-fed rats. Group II: short fasting period. Group III: long fasting period.

Group II, 70 rats starving 12–48 hours, of which 47 were examined after getting lævulose. In these the average rise of blood sugar was 18 mg. per 100 c.c.; in 19·2 p.c. less than 10, in 78·7 p.c. between 10 and 50, and in 2·1 p.c. more than 50 mg. per 100 c.c.

Group III, 46 rats fasting for 96 hours, of which 28 were examined after lævulose. In these the average rise of blood sugar was 41 mg. per 100 c.c., in 3·6 p.c. of them less than 10, in 71·4 p.c. between 10 and 50, and in 25 p.c. above 50 mg. per 100 c.c.

The blood-sugar curves of groups I and II (Fig. 1) show a moderate increase during the course of the experiment, whereas the curve of group III corresponds to a "high and prolonged blood-sugar curve"

[Vogt, 1932]. If the statements referred to above were correct groups I and II ought to have shown a good initial glycogen content in the liver and good formation and storage after lævulose, whereas a lack of liver glycogen in the resting rats and a deficiency of glycogen formation and storage should have been found in the third group.

The glycogen formation in the liver.

Group I. This consisted, as already mentioned, of rats which were fed on the eve of the experiment with lævulose and then kept starving. This procedure was adopted because when animals were killed without any starvation period or after a fasting period of four hours the glycogen in the liver varied very widely. By the method adopted livers were obtained which showed a good resting glycogen content. There was a considerable difference in the glycogen content of different series which may well be accounted for by difference in the season and in external circumstances. In any one series, however, the resting liver glycogen content was found to be of approximately the same value (Table II).

TABLE II. Resting glycogen content in the livers of rats.
Figures given in g. per 100 g. liver.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

Group I		Group II	Group III
Series S	4·90	0·14	0·26
	4·40	0·12	0·18
	5·79	0·25	0·36
Series J	2·41	0·32	0·16
	2·68	0·42	0·39
	2·60	0·07	0·52
	2·19	0·07	0·34
		0·08	0·54
Series W	6·16	0·06	0·54
	5·86	0·07	0·88
	7·03	0·15	0·08
Series V	3·19	0·04	0·15
	3·64	0·08	0·21
	3·60	0·06	0·53
		0·13	0·39
		0·14	0·12
		0·12	1·18
		0·08	0·13
		0·15	
		0·23	
		0·10	
		0·15	
		0·19	
		0·12	
		0·16	
	Average	0·14	0·39

After the standard dose of lævulose the average content of glycogen found in the liver (Table III) decreased in the course of the experiment. The most marked decrease was observed in series with the higher initial glycogen values, above 5 p.c. With an initial glycogen value below that figure there was a slight increase in the second and third hour, but a marked decrease occurred in the later hours of the experiment. As a control experiment water only was given. This was followed by a rapid and progressive decrease in liver glycogen. After the end of absorption (fourth hour of experiment) the glycogen in the liver was in every series actually less than at the beginning of the experiment.

TABLE III. Glycogen formation in the liver after 0·5 g. lævulose.
Figures given in g. per 100 g. liver above resting value.
+ indicates increase, − indicates decrease.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

	Group I		Group II		Group III	
Time	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats
1st hour	−0·64	3	+0·80	7	+0·43	5
2nd hour	−0·64	4	+2·05	9	+2·29	5
3rd hour	−0·40	3	+2·27	7	+3·05	5
4th hour	−1·57	3	+2·57	6	+3·74	4
5th hour	−1·71	3	+2·04	5	+4·01	5
6th hour	—	—	+1·89	5	+2·97	4

Group II. The liver glycogen after a short fasting period was found to be practically constant, usually below 0·2 and never exceeding 0·5 p.c. After the dose of lævulose the average amount of glycogen deposited in the liver was found to be 0·80 after 1 hour and 2·05 p.c. after 2 hours. The glycogen formation was generally smaller than the average in those rats in which absorption from the gut was low. After 4 hours 25 p.c. of the sugar absorbed was recovered as glycogen in the liver. The average absolute glycogen content after that period was 2·57 p.c. which is only slightly less than the absolute average figure of the first group (2·84) in spite of the considerable difference in the resting glycogen of these two groups.

Group III. The glycogen values after prolonged fasting were found to be practically constant and definitely higher than those in group II (Table II). A strong positive acetone reaction was present in the urine at the beginning of the experiment.

After the dose of lævulose the rate of glycogen formation in the liver during the first hour was poor, the average found being 0·46 p.c. in contrast with 0·80 in group II. At the end of the second hour it was the same as in group II (Table III); at the end of the fourth hour it was

3·74 p.c., which corresponds to 33 p.c. of the sugar amount absorbed, both these figures being higher than those of groups I and II.

The results given so far show that the amount of glycogen already in the liver does not determine the capacity of the liver to build up glycogen, as was suggested by Barrenscheen's experiments. The rats of group II, although they had but little glycogen in the liver, showed good glycogen formation, whereas the rats of group III with a somewhat higher resting glycogen value formed definitely less in the first hour. The store of glycogen built up after the end of the absorption period (fourth hour) was in both groups II and III better than in group I with a high initial glycogen value. Furthermore, the results do not confirm the explanation of the blood-sugar curve after lævulose hitherto given. According to the blood-sugar curves, groups I and II should have had a good store of glycogen in the liver to start with, and after lævulose have increased it considerably, whereas the reverse should have held for group III. It is, however, evident that group II had the smallest amount of glycogen in the liver before the dose of lævulose and group I the smallest amount after it; the best glycogen formation was found in rats of group III. When the increase of blood sugar is compared with the glycogen deposition in the liver (Table IV) it is seen that a high rise of blood sugar does not correspond to a small glycogen formation in the liver nor a small one with a large. The blood-sugar curve after lævulose has in these experiments no relation to the liver glycogen.

TABLE IV. Increase in liver glycogen in relation to the blood-sugar rise.
Increase in liver glycogen in p.c. of lævulose absorbed.

Increase in blood sugar mg. per 100 c.c.	1st hour		2nd hour		3rd hour		4th hour		5th hour		6th hour	
	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats
0–10	9	9	22	4	18	2	5	4	7	3	10	3
10–50	12	9	27	10	21	10	27	7	30	7	22	6
Above 50	13	1	31	2	42	1	27	2	24	1	—	—

The glycogen formation in the muscles.

Group I. The initial glycogen content of the muscles was approximately the same for all rats of this group of well-fed animals in spite of the considerable differences in the amount of liver glycogen in the different series (Table V). The average glycogen formation after lævulose was considerable; a comparatively large amount of glycogen was already deposited in the first hour after lævulose which was in all probability due to the breakdown of liver glycogen (Table VI).

Group II. The resting glycogen in the muscles of this group was low, as it was in the liver. After lævulose ingestion a good glycogen formation was found which increased every hour until the maximum was reached in the fourth hour of the experiment when absorption was finished. No appreciable difference is found in the average rate for the whole experiment between groups I and II (Tables V and VI).

TABLE V. Resting glycogen content in the muscles of rats.
Figure given in g. per 100 g. muscle weight.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

	Group I	Group II	Group III
	0·31	0·16	0·20
	0·31	0·17	0·24
	0·36	0·14	0·24
	0·32	0·14	0·36
	0·38	0·15	0·38
	0·31	0·20	0·19
	0·22	0·18	0·20
	0·40	0·15	0·32
	0·26	0·28	0·21
	0·31	0·22	0·27
	0·27	0·15	0·26
	0·41	0·19	0·12
	0·26	0·22	0·23
	0·26	0·18	
	0·32	0·16	
	0·24		
	0·28		
Average	0·31	0·18	0·25

TABLE VI. Glycogen formation in the muscle after lævulose.
Figures given in g. per 100 g. muscle above resting value.
+ indicates increase, − indicates decrease.

Group I, well-fed rats. Group II, short fasting period. Group III, long fasting period.

	Group I		Group II		Group III	
Time	Glycogen	No. of rats	Glycogen	No. of rats	Glycogen	No. of rats
1st hour	+0·12	3	+0·06	6	−0·01	5
2nd hour	+0·06	5	+0·08	8	+0·06	5
3rd hour	+0·13	5	+0·11	4	+0·09	5
4th hour	+0·13	5	+0·18	6	+0·07	4
5th hour	+0·13	3	+0·08	3	+0·05	4
6th hour	+0·12	2	+0·12	5	−0·02	4
Average	+0·12	23	+0·10	32	+0·04	27

Group III. The average initial glycogen content in this group held, as in the case of the liver, an intermediate position between the figures for groups I and II. No glycogen formation occurred during the first hour; the maximum was found after 3 hours and was about half as much as the maximum in group II. At each hour the figures are lower than

those for either group I or II, the average for the whole experiment being only one-third (Tables V and VI).

It must be noted that the increase of muscle glycogen is small like the initial glycogen content. The variations observed in the resting glycogen content in the liver, especially if this is low, do not affect the results after lævulose because comparatively large quantities of glycogen are newly formed. The same variations, when they occur as they do, in the resting muscle glycogen are much more liable to affect the result owing to the comparatively small amount of glycogen formed. The number of animals, however, from which these averages are calculated is such that the figures certainly indicate a diminished power of building up glycogen in the muscles of rats that have fasted long (group III).

There is a definite relationship between the formation of muscle glycogen and the corresponding increases in blood sugar (Table VII). Rats showing a marked increase in blood sugar (above 50 mg. per 100 c.c.) show little if any formation of glycogen in the muscle; the amount formed is largest when the blood sugar rises least. The capacity of the muscle to build up glycogen seems, therefore, to have a definite relation to the blood-sugar curve. The blood sugar after lævulose is high when the muscles cannot form glycogen.

TABLE VII. Increase in muscle glycogen in relation to the blood-sugar rise after 0·5 g. lævulose meal.

Figures given in g. per 100 g. muscle weight above resting value and in mg. per 100 c.c. blood. Where the − sign appears there is no increase but a diminution.

Increase in blood sugar	1st hour		2nd hour		3rd hour		4th hour		5th hour		6th hour	
	Gly-cogen	No. of rats	Gly-cogen	No. of rats	Gly-cogen	No. of rats	Gly-cogen	No. of rats	Gly-cogen	No. of rats	Gly-cogen	No. of rats
0–10	0·12	5	0·10	5	0·12	4	0·15	5	0·17	2	0·16	3
10–50	0·03	9	0·06	8	0·11	10	0·11	6	0·09	6	0·03	7
Above 50	−0·08	1	0·01	3	0·02	1	0·03	2	0·05	2	—	—

The effect of thyroxine.

The following experiment points to a similar conclusion. Abderhalden and Wertheimer [1930] showed that after thyroxine the livers of rats are unable to form glycogen. A series of seven rats were, therefore, injected daily with 0·5 mg. thyroxine (Schering) for 7 days. The animals were then kept fasting 12 hours before giving the lævulose. The liver glycogen of the fasting rats was very low, 0·06 p.c. Following lævulose ingestion there was hardly any increase, the maximum after 3 hours being 0·16 p.c. The muscle, however, the initial glycogen content of

which was also very low (0·09 p.c.), had risen after 3 hours to a maximum, 0·25 p.c., an increase similar to that observed in rats of group II. There was no marked rise in the blood-sugar level. This result is very much against the old explanation that the failure of the liver to form glycogen accounts for the rise of blood sugar after lævulose, while on the contrary the relation between glycogen formation in the muscles and blood sugar is the same as that in the experiments recorded above. With larger doses of thyroxine the blood sugar tends to rise considerably after lævulose [Kugelmann, 1930] and the glycogen formation in the muscles of rats becomes poor [Abderhalden and Wertheimer, 1930].

Summary.

1. It has been commonly held that (*a*) the power of the liver to form glycogen is impaired when its store of glycogen is low; and that (*b*) an excessive rise of blood sugar after taking lævulose is due to the inability of the liver to form and store glycogen. The experimental basis of these opinions has been critically examined.

2. The power of the liver to form glycogen has been shown not to be impaired when its store of glycogen is low.

3. An excessive rise of blood sugar after lævulose has been found to be associated neither with a low glycogen store nor with inability of the liver to form and hold glycogen.

4. On the other hand such a rise of blood sugar after lævulose was associated with little or no glycogen formation in the muscles.

5. The results suggest that inability, not of the liver, but of the muscles to build up glycogen is at least one factor of importance in an excessive rise of blood sugar after lævulose. The clinical observation that such a blood-sugar curve is given in disease of the liver is not disputed, nor is it incompatible with the above results if the anabolism of glycogen in the muscles is influenced by the metabolic changes which carbohydrates undergo in the liver.

I wish to express my thanks to Prof. von Bergmann for many kindnesses shown to me while working at the Charité Krankenhaus, Berlin, and for facilitating this investigation in every way. I am also extremely indebted to Dr A. F. Hurst for his encouragement and advice which has enabled me to correlate my researches and present them in this form; and to Mr Spurrell and Dr S. L. Simpson for very helpful criticism.

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THE CARBON DIOXIDE BALANCE BETWEEN THE MATERNAL AND FŒTAL BLOODS IN THE GOAT.

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Introduction.

The possibility of carbon dioxide secretion at or in the placenta was not entertained by the earlier investigators, although the same question with regard to oxygen was most seriously debated [Zuntz, 1877; Cohnstein and Zuntz, 1884; Charpentier and Butte, 1888; Butte, 1893; Bohr, 1909]. Subsequent demonstration of the high diffusion coefficient of carbon dioxide [Krogh, 1919] indicated that passive exchange of carbon dioxide should be ample to provide for its elimination from the fœtus. Huggett's "differential tonometry" [1927] pointed to a passive diffusion of CO_2 across the placenta of the goat, but the data given are few and not entirely conclusive.

Granting for the moment the probability that the elimination of CO_2 from the mammalian embryo does not involve secretory work, there remains a second problem with regard to the avoidance of acidosis in the embryo. It is well known that the pregnant female is relatively acidotic and that in late pregnancy a degree of acidity is attained which would be considered dangerous in a non-pregnant subject [Williamson, 1923]. If there is always a CO_2 gradient from the fœtus to the mother, the fœtus might be expected to show an even more pronounced acidosis.

Material for the study of these questions was available at Cambridge during the investigation of the fœtal oxygen relations in the goat [Barcroft, 1933], and the data presented here were obtained from the bloods of the animals used in the experiments on oxygen exchange.

Methods.

The anæsthesia, operative technique and collection of the blood in these goat experiments have been described in detail by Barcroft [1933]. All blood was drawn into syringes containing oxalate and

fluoride solution which was in tonometric equilibrium with room air; the oxalate-fluoride solution was 10 p.c. of the final volume in the syringe.

Determinations of CO_2 and O_2 contents of the bloods were made in duplicate with the van Slyke apparatus immediately after drawing. A second blood portion was equilibrated with room air for the determination of the oxygen capacity, and the rest of the blood stored in a tonometer with a large gas phase in which the partial pressures of CO_2 and O_2 were each about 50 mm. Hg. Blood samples from this tonometer were then equilibrated at 37° C. in the new Barcroft tonometers [Barcroft, 1934] in gas mixtures (calculated to give about 50 p.c. oxygen saturation) over the general range 30–60 mm. partial pressure of CO_2. The blood gas analyses were done in duplicate on 0·2 c.c. portions using the standard van Slyke procedure. At the end of the day a second determination of oxygen capacity was made in most cases; these showed little or no change.

In a few cases blood drawn directly from the animal was analysed for carbon dioxide tension by means of a modification of Krogh's [1908] bubble method.

Carbon dioxide contents and tensions.

In the earlier stages of pregnancy, fœtal blood sufficient for study of the CO_2 relations was not obtained; however, in two cases carbon dioxide tensions were determined by the bubble technique on blood from the maternal jugular vein of the anæsthetized goat after laparotomy. Tensions of 41 and 48 mm. Hg were found; a later determination on the second goat gave a CO_2 partial pressure of 53 mm. Hg with indication of slight anoxæmia after 85 min. of profound anæsthesia and very extensive operative manipulation. These results indicated that maternal CO_2 elimination was well maintained under the experimental procedure.

In two other cases bubble determinations were successful with both maternal and fœtal bloods (from the uterine and umbilical veins respectively). The results are given in Table I; in both cases the carbon dioxide tension on the fœtal side of the placenta was slightly higher than on the maternal side.

The other four sets of figures in Table I were obtained from values for CO_2 contents and the individual CO_2 dissociation curves for the same bloods; the method of obtaining these figures will be apparent from an inspection of Figs. 1–4. The six cases show clearly that under the

TABLE I. CO_2 tensions in the blood as drawn from the animal. Values in mm. Hg.

Date	No.	Days of pregnancy	Maternal	Fœtal
April 18	8*	85	45	47
May 1	10*	101	46	52
May 5	11†	106	40·8	40·5
May 12	12†	113	41·5	48·5
May 22	14†	126	38·3	51·5
May 31	16†	(105)‡	44·7	44·5

* Determined by bubble analysis.
† Determined from CO_2 content and CO_2 dissociation curve for the same blood.
‡ Approximate, calculated from weight of fœtus.

experimental conditions CO_2 elimination from the fœtus is effectively maintained without the appearance of any secretory mechanism.

The gas contents of the fœtal blood as drawn from the animals may be compared with values obtained by Huggett [1927] and Cohnstein and Zuntz [1884] for goats.

TABLE II. Mean gas contents of fœtal arterial blood (from the umbilical vein) as drawn from the animal.

Reference	p.c. O_2 saturation	O_2, vol. p.c.	CO_2, vol. p.c.
Present determinations	64·1	8·27	47·0
Huggett [1927]	45·0	7·96	29·9
Cohnstein and Zuntz [1884]	—	6·3	40·5

THE ALKALI RESERVE.

The carbon dioxide dissociation curves for the fœtal and maternal bloods are shown in Figs. 1–4; they show uniformly a markedly higher alkali reserve in the fœtal bloods than in the respective maternal bloods. That this is not due simply to the smaller cell volumes in the fœtal bloods is shown by the lines for the plasmas (solid lines in the figures) which were calculated from the oxygen capacities and the whole-blood dissociation curves by means of the line charts of van Slyke and Sendroy [1928, see Peters and van Slyke, 1932, p. 289].

The propriety of the use of van Slyke's line charts for these calculations is shown in Table III by the agreement between cell volumes calculated in this way and the cell volumes observed with the hæmatocrit[1].

The average fœtal whole blood contained 22 p.c. more CO_2 than the corresponding maternal whole blood at the same CO_2 tension; the fœtal plasma averaged 18·6 p.c. higher in CO_2 capacity than the maternal

[1] By the kindness of Dr A. St G. Huggett.

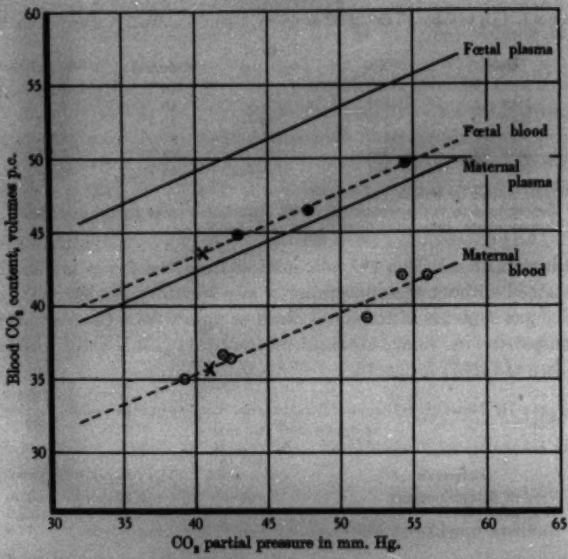

Fig. 1. Carbon dioxide dissociation curves for fœtal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 47 mm. Hg throughout. The crosses show the CO_2 contents of the bloods as drawn from the animal. Goat No. 11, May 5, 106 days' pregnant.

TABLE III. Cell volumes calculated from oxygen capacity and comparison with hæmatocrit values.

Date	Goat No.	Blood	Cell vol. p.c. of whole blood: Calculated	Observed
April 18	8	Fœtal	38·4	40
May 1	10	Fœtal	23·1	22
May 5	11	Maternal	39·2	
		Fœtal	27·0	
May 12	12	Maternal	37·1	
		Fœtal	31·7	32
May 22	14	Maternal	40·2	
		Fœtal	27·8	29
May 31	16	Maternal	27·8	
		Fœtal	25·0	
May 18	Control 13		44·2	
May 16	Control C_2	Before anæsthesia	28·1	
		15 min. „	33·0	
		75 min. „	35·0	
May 29	Control	Before anæsthesia	20·8	
		90 min. „	24·9	

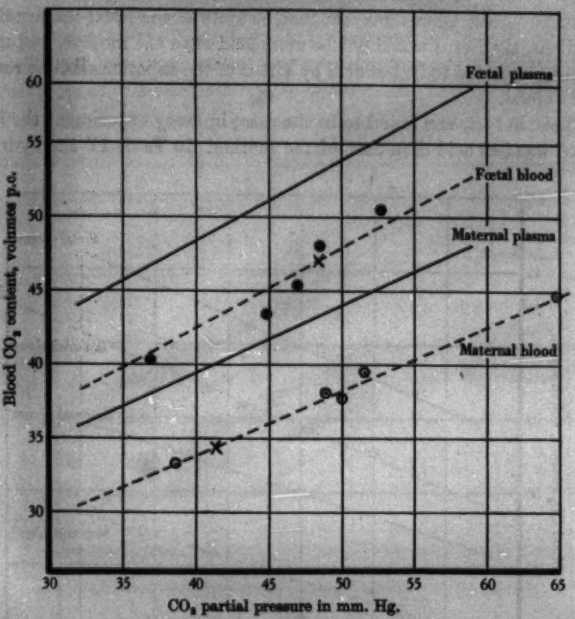

Fig. 2. Carbon dioxide dissociation curves for fœtal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 43–48 mm. Hg. The crosses show CO_2 contents as drawn. Goat No. 12, May 12, 113 days' pregnant.

TABLE IV. Blood hydrogen ion concentrations at 37°.

Date	Goat No.	Blood	pH calculated	pH observed*
May 5	11	Maternal	7·26	7·26
		Fœtal	7·34	7·38
May 12	12	Maternal	7·22	7·25
		Fœtal	7·30	7·31
May 22	14	Maternal	7·23	7·14†
		Fœtal	7·27	7·30
May 31	16	Maternal	7·33	7·33
		Fœtal	7·35	7·34
May 16	Control C_2	Before anæsthesia	7·37	
		15 min. „	7·31	
		75 min. „	7·35	
May 18	Control 13		7·38	
May 29	Control	Before anæsthesia	7·50	
		90 min. „	7·41	

* Values obtained with the glass electrode and quoted here by the kindness of Dr R. E. Havard and Mrs S. Dickinson.

† May be erroneous.

plasmas. These figures indicate that, in spite of the fœtal-maternal CO_2 gradient, the fœtus would not be more acid than the mother, and might even be expected to be less acid by virtue of the superior alkaline reserve of its blood.

This, in fact, was found to be the case; in every experiment the fœtal blood was less acid than that of the mother. In Table IV the hydrogen

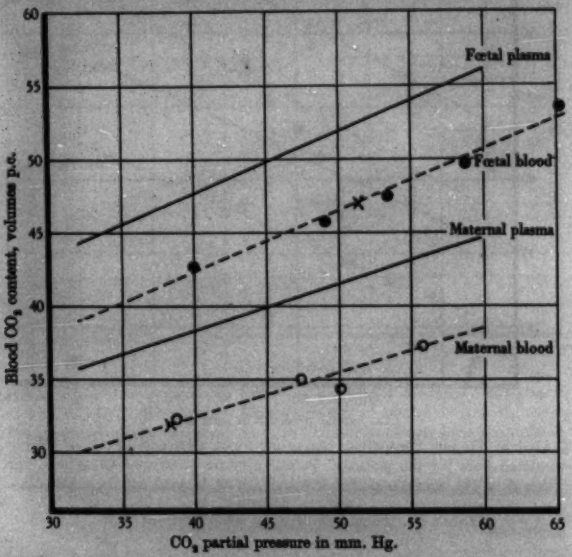

Fig. 3. Carbon dioxide dissociation curves for fœtal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 43–48 mm. Hg. The crosses show CO_2 contents as drawn. Goat No. 14, May 22, 126 days' pregnant.

ion concentrations are given for the experimental animals as well as for three non-pregnant female goats. The calculated *p*H values were obtained by the use of the Henderson-Hasselbalch equation:

$$pH = pk + \log [BHCO_3] - \log [H_2CO_3],$$

taking $pk = 6{\cdot}10$ [Hastings, Sendroy and van Slyke, 1928]. For this purpose the line charts of van Slyke and Sendroy given by Peters and van Slyke [1932, pp. 289, 294] were used in most cases.

The excellent agreement between the calculated values and the values obtained by the glass electrode is a triumph for the Henderson-Hasselbalch equation under severe conditions, and warrants more extensive use of this calculation with hitherto uninvestigated bloods.

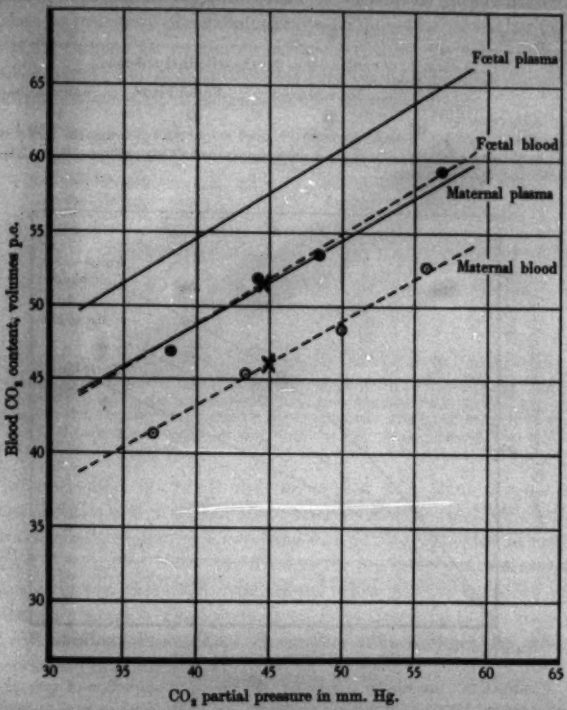

Fig. 4. Carbon dioxide dissociation curves for fœtal and maternal whole bloods with calculated lines for the plasmas. Partial pressure of oxygen 30 mm. throughout. The crosses show CO_2 contents as drawn. Goat No. 16, May 31, about 110 days' pregnant.

Discussion.

The present series of experiments reveals a nicely adjusted system which provides for elimination of fœtal carbon dioxide without requiring secretory work and at the same time safeguards the hydrogen ion con-

centration of the fœtal blood. This is not attained, however, without some sacrifice—but the sacrifice is on the part of the mother. Both in alkali reserve and in *p*H the maternal blood alters very much from the normal goat blood, but the fœtus is protected and its blood is much more nearly normal than the mother's. This is brought out in Table V in which average values for whole blood are tabulated.

TABLE V. Average value for anæsthetized animals.

	Normal goat	Fœtal blood	Maternal blood
CO_2 capacity*	51	47	38
*p*H	7·38	7·32	7·26

* At half-saturation with oxygen and partial pressure of CO_2=45 mm. Hg. Values are in volumes p.c. for whole blood.

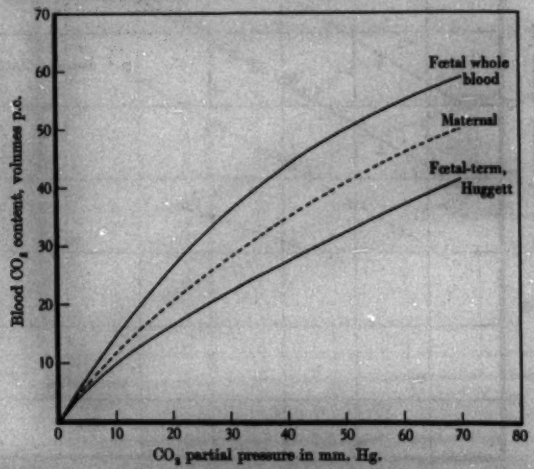

Fig. 5. Complete CO_2 dissociation curves for whole blood. Comparison of average fœtal and maternal bloods at 3½ months' pregnancy and fœtal blood at term as calculated from Huggett [1927]. All values for oxygen partial pressure 47 mm. Hg.

In the course of fœtal development there seems to be a general depletion of the alkali reserve; Williamson [1923] observed a continuous fall in the CO_2 capacity of the blood of pregnant women with the advance of pregnancy. For the goat, figures obtained here for 3½ months' pregnancy may be compared with values calculated from Huggett's data for the fœtal blood at or very near term (Fig. 5). Even allowing for possible

differences in states of nutrition, etc., in the two sets of animals, there is a much lower alkali reserve at term than (roughly) six weeks earlier. It is of interest to recall that Zangemeister and Meissl [1903] observed a progressive decrease in the osmotic pressure of the blood of women in pregnancy.

The gas transport properties of fœtal blood as indicated in the work here at Cambridge are compared in Table VI with the values calculated from Huggett. The goat fœtus at term shows a much higher O_2 capacity, but a much lower CO_2 capacity than the 3½-month fœtus.

TABLE VI. Plasma CO_2 capacity of fœtal bloods as indicated by CO_2 content at a CO_2 partial pressure of 45 mm., when the partial pressure of oxygen is about 43 mm. Hg.

Fœtuses 100–130 days old. Present determinations		Fœtuses at or near term. Calculated from Huggett [1927]	
O_2 capacity vol. p.c.	Plasma CO_2 capacity vol. p.c.	O_2 capacity vol. p.c.	Plasma CO_2 capacity* vol. p.c.
11·7	57·5	15·5	27·4
12·4	51·3	17	30·2
12·9	49·8	18	44·5
14·1	51·1	19	37·4
—	—	19	48·4
Means 12·8	52·4	17·7	37·6

* Calculated from partial dissociation curves constructed from Huggett's tonometric data, corrected to O_2 partial pressure of 45 mm. from Huggett's figures for the effect of oxygen on the CO_2 capacity of the blood in his experiments. The transformation from whole blood to blood plasma was made by means of the line chart given by van Slyke and Sendroy [1928].

Unfortunately, Huggett did not collect data from which a comparison of fœtal and maternal alkali reserves may be made. In fourteen cases of normal pregnancy, Losee and van Slyke [1917] found maternal plasma CO_2 capacity below the average for non-pregnant normals in all cases, and in ten cases the values were below what is regarded as the "minimum normal." They drew blood from four infants 2–3 days after birth and obtained an average CO_2 capacity of 53 volumes p.c., while the corresponding mothers averaged 50.

Williamson [1923] found the average CO_2 capacity of blood of infants at birth to be 10 p.c. higher than the average for their mothers. He observed that the alkali reserve of the mothers' blood rapidly rises after parturition and is back almost to normal within 10 days. Weissmann-Netter [1925] found an even greater superiority in CO_2 capacity of the blood of normally delivered new-born infants; similar results were obtained from fœtal and maternal bloods in four Cæsarian deliveries [Levy-Solal, Weissmann-Netter and Dalsace, 1926].

Opposed to this body of evidence are the results obtained by Bell,

Cunningham, Jowett, Millet and Brooks [1928] who studied two Cæsarian section deliveries and a few cases of normal delivery; they found the CO_2 capacity of the fœtal whole blood to average about 5 p.c. less than the maternal blood, and believed this to be due to the high lactic acid level (average 68 mg./100 c.c.) found to characterize the fœtal blood.

There is little evidence as to the chemical substances responsible for the difference in physiological buffering power between the maternal and fœtal bloods. Unfortunately, the analytical data in the literature in most cases report simply concentrations of the various substances in the whole blood; in view of the pronounced differences in the cell volumes of maternal and fœtal bloods, comparison is difficult.

In man, at least, there is practical identity between fœtal and maternal blood in respect to total osmotic pressure and chloride, while total protein nitrogen is slightly higher in the maternal blood [Needham, 1931, 3, 1531–3; Füth and Wirz, 1929].

In the goat Albano [1903] reported the fœtal blood to have about 4 p.c. higher osmotic pressure than the maternal blood, but Albano's similar statement with regard to the cow is opposed by the studies of Ubbels [1901] and Grünbaum [1904] who found the same osmotic pressure in both fœtal and maternal blood.

All investigators [see Needham, 1931, p. 1531] agree that the concentrations of calcium and inorganic phosphorus are higher in fœtal blood than in maternal blood (in man, dog, rabbit, cow and pig). Sodium and potassium have been variously reported as identical, higher in maternal and higher in fœtal blood; at first sight the balance of evidence indicates a preponderance of potassium on the fœtal side, but when calculations are made on the basis of Na and K in the *plasma*, the values are very nearly equal [Von Oettinger, 1926; Edelstein and Yllpö, 1921; Bąkwin and Rivkin, 1927; Krane, 1930].

In the present series of experiments a few measurements of serum refractive index gave slightly higher values for the maternal blood, indicating a probable higher protein concentration in the mother. On the basis of present knowledge it would seem that part of the higher CO_2 capacity of the fœtal blood is due to the excess of phosphates and calcium compounds in it; some other factors, however, must be involved.

Summary.

Measurements of carbon dioxide tension in fœtal and maternal blood of the goat showed that passive diffusion would suffice for the fœtal CO_2 elimination under the conditions of the experiments.

The fœtal blood has a much greater CO_2 capacity than the maternal blood and this is due to a greater alkali reserve in the plasma.

By virtue of this higher alkali reserve the fœtal blood is less acid than the maternal blood, in spite of the fact that the fœtal CO_2 content is always much larger.

Both in blood *p*H and in alkali reserve the goat fœtus is more nearly normal than its mother.

Prof. J. Barcroft made possible the undertaking of the present study and his stimulating interest secured its completion.

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A SPECTROSCOPIC METHOD FOR THE STUDY OF HÆMOGLOBIN IN DILUTE SOLUTIONS.

By F. G. HALL.

(*From the Physiological Laboratory, Cambridge.*)

(*Received November 17th*, 1933.)

Introduction.

The comparison microspectroscope has been used by several investigators for the study of hæmoglobin [Bürker, 1911]. Krogh [1919] described a method and Krogh and Leitch [1919] used such an instrument to study hæmoglobin in fishes' blood. More recently the microspectroscope has been employed in the study of respiratory pigments by Keilin and Hill. The author is indebted to both Dr Keilin and Mr Hill for suggestions which have led to the use of this instrument as herein described. The following description involves no new use of the microspectroscope. The purpose of this paper is to outline a procedure rather than to describe the use of any particular instrument. Perhaps in this way it may serve others who may be interested in a relatively simple technique for making oxygen dissociation curves on limited quantities of blood.

The microspectroscopic comparator.

A Zeiss microspectroscopic eyepiece was arranged as shown in Fig. 1. A colorimeter plunger was attached to the tube of the eyepiece. A colorimeter cup was held in a fixed position some centimetres below. Between the fixed cup and the fixed plunger a movable cup (C_1) and a movable plunger (P_1) could be raised or lowered by means of a rack and pinion. Thus the sum of the depths of solutions in movable and fixed cups was always constant regardless of the position of the movable cup and plunger. A depth of 20 mm. was found a convenient one. Consequently it will be seen that when a solution of fully oxygenated hæmoglobin was placed in one cup and of fully reduced hæmoglobin in the other, one could obtain a spectrum of hæmoglobin representing any degree of oxygenation by

simply varying the relative depth of the solutions in the two cups. The ratio of depths of the two hæmoglobin solutions to one another was read on a vernier scale. A tonometer (T) containing the hæmoglobin solution under investigation was placed in front of the side opening of the eyepiece

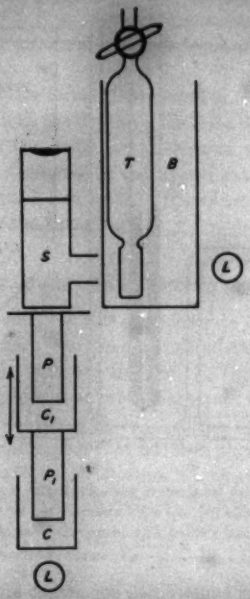

Fig. 1. Diagram to show the relative positions of the spectroscopic eyepiece (S), the tonometer (T), the colorimeter plungers (P, P_1) and the colorimeter cups (C, C_1). The tonometer was kept in a glass water bath (B). Two lights were fixed in the position (L) and their intensity regulated with resistances so that the comparison spectra were of equal intensity.

(Fig. 1). In this manner the two spectra could be matched and the degree of oxygen saturation of the unknown determined.

A nitrogen container (Fig. 2) was constructed by inverting a large bottle and filling it about one-third full with a concentrated solution of sodium hydrosulphite. Nitrogen from a supply tank was bubbled through the hydrosulphite solution and allowed to stand for some hours before using. Samples were withdrawn from time to time and analysed with a

Haldane gas analysis apparatus. No detectable quantity of oxygen was ever found. A manometer (M) was arranged to show the pressure within the nitrogen container or within the tonometer as desired. This could be accomplished by manipulation of the stopcock (S_2).

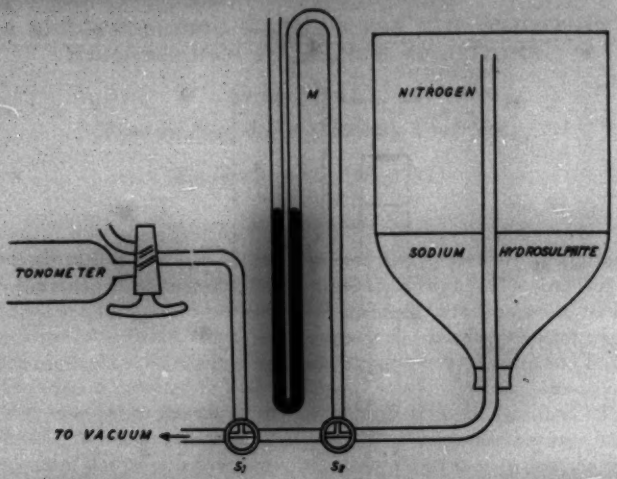

Fig. 2. Diagram to show how tonometers were filled with gas mixtures of known oxygen pressures. Two stopcocks (S_1, S_2) were manipulated so that the tonometer could be alternately evacuated and refilled with nitrogen. Pressures were read on manometer (M).

The tonometer was constructed somewhat after that used by Redfield [1930], except that the cell was blown from one piece of glass. The cell was approximately 2 cm. in diameter and 1 cm. deep. The total capacity of the tonometer was about 35 c.c.

Procedure.

3 c.c. of a dilute hæmoglobin solution were placed in the tonometer. The tonometer was then evacuated three times, refilling with nitrogen each time. By gentle shaking the dissolved gases could be removed from the solution and the hæmoglobin fully reduced. Fig. 2 shows the position of the stopcocks in relation to the vacuum line, nitrogen supply and tonometer. It was found more convenient to draw the nitrogen from the

manometer by manipulation of a stopcock (S_3) than directly from the nitrogen container. After the last evacuation the pressure in the tonometer was left somewhat below atmospheric, and a measured quantity of air was admitted from a gas burette. The temperature of the air in the gas burette and the atmospheric pressure were noted. The tonometer was then shaken for 10 min. in a constant temperature bath. It was then quickly removed to the glass water bath in front of the spectroscope and a reading taken at once.

The standard hæmoglobin solution placed in the colorimeter cups was prepared as follows. Since the depth of the solution in the cups was twice that in the tonometer the hæmoglobin solution was diluted to one-half that in the tonometer. One portion was placed in the lower cup and about 5 mg. of sodium hydrosulphite added. The other portion was saturated with air and placed in the upper cup.

Calculations of the oxygen tensions.

If $V_{\text{Ton.}}$ is the capacity of the tonometer in c.c., V_B the volume of hæmoglobin in c.c. which has been admitted into the tonometer and freed of dissolved and combined oxygen, V_A the quanity of air in c.c. introduced into the tonometer at room temperature (T_1) and atmospheric pressure (P) in mm. of Hg, and $V_{O_2(c+d)}$ the c.c. of oxygen which is combined with hæmoglobin plus that which is physically dissolved in the hæmoglobin solution after equilibration at temperature T_2, then the partial pressure of oxygen (pp) in mm. of Hg will be:

$$pp = \frac{0{\cdot}2094\,V_A - V_{O_2(c+d)}}{V_{\text{Ton.}} - V_B} \times \frac{T_2}{T_1} \times P.$$

Since the quantity of physically dissolved and combined oxygen is small, $V_{O_2(c+d)}$ may be approximated without significant change in values for oxygen tensions.

It was thought desirable to compare the spectroscopic method with the van Slyke-Neill manometric method using similar samples of blood. Hæmoglobin from sheep blood was prepared free from stroma protein by the use of ether and salt as described by Adair [1925] and kept in a $M/15$ phosphate buffer solution of pH 6·8. One portion was diluted fifty times with the phosphate buffer solution. A sample of whole blood after having been hæmolysed with a minimal quantity of distilled water was diluted fifty times with the phosphate buffer solution of pH 6·8. The concentrated hæmoglobin solution was used for the van Slyke technique and equilibrated in Barcroft tonometers. The two

diluted samples treated in a similar manner described above were analysed with the spectroscopic comparator. Three dissociation curves thus obtained are shown in Fig. 3. The two methods seem to check satisfactorily

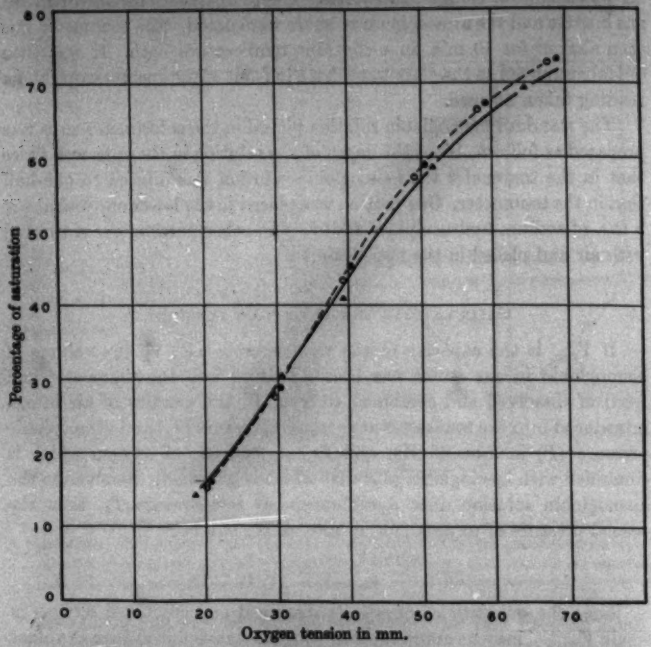

Fig. 3. Three dissociation curves obtained by three different methods. Sheep blood at pH 6·8 was used.

▲ represent analyses made with a van Slyke manometric apparatus on a hæmoglobin solution prepared by the Adair method.

o represent analyses made with the comparison spectroscope on whole blood hæmolysed and diluted fifty times with phosphate buffer solution $M/15$ and pH 6·8.

• represent analyses made with the comparison spectroscope on the hæmoglobin solution prepared by the Adair method and diluted fifty times with the phosphate buffer solution.

with one another. There appears to be no difference in curves obtained with hæmolysed whole blood and with hæmoglobin prepared by the Adair method.

There are certain limitations to the spectroscopic method. Determinations of oxygen saturation between 0 and 20 p.c. or between 80 and 100 p.c. cannot be made accurately. Also an error is introduced if appreciable methæmoglobin is formed since the absorption bands of methæmoglobin are quite similar to those of oxyhæmoglobin.

The advantages of the method are: a small quantity of blood is required and it is apparently not necessary to prepare a purified hæmoglobin solution. The *p*H, temperature, and oxygen tension can be controlled accurately and easily.

SUMMARY.

A spectroscopic method is described whereby dissociation curves can be made on small quantities of hæmoglobin.

I wish to thank Prof. Barcroft and Mr Adair for laboratory facilities and for many helpful suggestions.

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PROCEEDINGS
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PHYSIOLOGICAL SOCIETY,
October 21, 1933.

Observations on the electrical activity of the cortex.

By E. D. Adrian and B. H. C. Matthews.

The electric changes produced by injury to the cerebral cortex show that there is a considerable uniformity in time relations in the different nervous elements. In rabbits under urethane puncture of the grey matter with an exploring electrode leads, as a rule, to an outburst of rhythmic oscillations of the order of 0·1 millivolt, due to the synchronous activity of many neurones. These outbursts have remarkably constant features. They consist of a rapid train of waves with the sinusoidal form characteristic of synchronous discharges in general. The initial frequency may be as high as 120 a sec., but often a second or more elapses before full synchronization is reached and the frequency then lies between 70 and 50 a sec. The discharge continues at this rate for 5 to 20 secs. Eventually the waves become smaller and less regular, with or without a slight fall in rhythm, and all trace of regularity disappears when the rate has fallen below 40 a sec. Outbursts of this kind may be produced in the superficial layers of the cortical grey matter, in the deeper layers and in the basal ganglia. Occasionally the regularity persists at much lower frequencies and sometimes very large isolated waves appear from time to time in the course of a regular discharge; but the typical effect of injury is the discharge at 70–50 a sec. The same effect is found in the cat under urethane.

The rhythmic potential oscillations caused by strychnine, picrotoxin, etc. have been found by Fischer [1933] to have the same characteristic frequency, though in these outbursts the number of units in action must be much greater and isolated waves or groups of waves are more common. It is arguable that injury and strychnine excite only a particular class of neurone, but in all those which are excited there can be no great difference in time relations in so far as rhythmic activity is concerned.

Simultaneous records made from three points in the cortex show that an injury discharge often involves a very wide area, a point 9 mm. from the seat of injury giving a pulsation which corresponds, wave for wave, with that from the injured point, though often with some phase difference. The amount of spread varies widely. In the absence of any kind of stimulus

large, irregular potential disturbances of much longer duration are found in all parts of the cortex. These usually occur (in the rabbit) at intervals of 1–4 sec., not related to respiration or the heart beat. They often appear almost simultaneously at points 6 or 9 mm. apart, though there may be periods of complete lack of correspondence. The slow disturbances are usually accompanied by small, rapid oscillations of the type seen in the injury discharge and due presumably to synchronous pulsation in a group of neurones. There is no clear agreement in the rapid waves at points more than 3 mm. apart. Thus the detailed pattern of activity may vary from one point to another although the general pattern is the same over a wide area. With intense activity, however, the neurones over very large areas may act in complete unison.

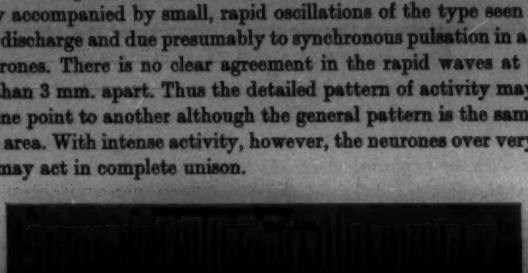

Fig. 1.

A. Injury discharge recorded at two points 6 mm. apart in parietal cortex. Electrodes 1 mm. deep, injury due to puncture at a point 3 mm. lateral to one electrode. Corresponding waves.

B. Records from three points in line at intervals of 3 mm. on parietal cortex. Rabbit under urethane, no stimulus. ·1 mf. condensers in amplifiers to show detail of rapid waves. Time marker gives ·05 sec.

Our experiments have been confined to animals under urethane anæsthesia, but the results seem to agree in the main with those of others who are investigating the electric changes in the cortex [cf. Bartley and Bishop, 1933; Fischer, 1932, 1933; Kornmüller and Tönnies, 1933, etc.].

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Electric stimulation of the cochlea. By B. Fromm, O. Nylén and Y. Zotterman. (*Caroline Institute, Stockholm.*)

Adrian has pointed out [1931] that the Wever and Bray effect is easily led off from the round window in the cat. We repeated this experiment in November 1932 and obtained the same result. Since then we have attempted to record the effect in man, using patients where the round window is accessible through large perforations in the drum. These experiments have been so far unsuccessful, though they were made with the same amplifying arrangements which we used successfully in the cat. In the course of this work, however, we thought it worth while to try the converse experiment of producing a rapidly fluctuating potential in the neighbourhood of the round window to see if this would give rise to the sensation of sound.

Concerts from a gramophone pick-up were amplified and led to the ear by way of a condenser and volume control. The electrodes were arranged in the following way. A 0·25 mm. enamelled copper wire, with the end bared and bent round a small piece of cotton wool was introduced through the auditory canal and put in contact with the lateral wall of the labyrinth. The other electrode was a syringe needle inserted in the neck. The potentials applied to the electrodes were reassured by a Weston A.C. instrument.

Out of eighteen patients we succeeded in five in producing definite sensations of sound. These could distinguish tunes played on the gramophone (which made no audible sound) and two could distinguish speech.

In all cases the positive result was only obtained when the electrode was in the neighbourhood of the windows, and in no case was the result obtained with less than 30 volts. When applying a voltage of more than 60 volts unpleasant excitation of the facial and glossopharyngeal nerves and chorda tympani as well as n. vestibularis was frequently observed.

At the present stage of the investigation it is of course difficult to enter into any discussion about the real nature of the effect described above. It is possible that it is caused by electrostatic effects in the ear which are transformed into mechanical vibrations, thus eliciting a natural stimulus of the receptors of the cochlea. There was, however, no change in the effect when the electrode used was bare or when a piece of cotton wool moistened with salt solution was attached to the end. Future investigations in regard to the upper limit of sound perception are planned. As far as our experience now goes notes from 200 to 3500 were perceived by one subject.

The fact that with the voltage amplitudes applied by us the effect is brought out only when the electrode is placed in the neighbourhood of the windows suggests that it is probably due to a direct stimulation of the cochlear apparatus.

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The relation of corpora lutea to gestation and anterior pituitary in some lower vertebrates. By J. T. CUNNINGHAM and W. A. M. SMART. (*Department of Pharmacology, London Hospital Medical College.*)

It has been shown that injection of extract of anterior pituitary in the South African frog, *Xenopus*, results in ovulation within 24 hours. The same injection in mammals causes the formation of corpora lutea which in normal reproduction are developments of the discharged follicles. We exhibit sections of the ovary after ovulation induced by anterior pituitary injections in *Xenopus*. In this case the discharged follicles undergo no hypertrophy, but are at once reduced and absorbed, as seen by comparing the ovaries immediately after ovulation, and after an interval of some days. No ovulation was produced by similar injections in *Rana esculenta*, *R. temporaria*, or *Bufo calamita* (Natterjack toad). On the contrary the ovary in these species underwent reduction in size (involution).

We were particularly interested in the question of the relation between gestation and corpora lutea in the lower vertebrates. We have therefore compared the history of discharged follicles in the viviparous forms—*Anguis* (Slow-worm), *Zootica* (viviparous English lizard)—with that found in *Lacerta*, in which the eggs are expelled from the oviduct after ovulation. We found that the discharged follicle persisted without reduction in the viviparous form up to the latest stage of embryonic development in the oviduct which we obtained. The discharged follicles in those reptiles therefore which exhibit gestation are true corpora lutea, and their persistence is evidently associated with the development of the embryos in the oviduct.

In a specimen of *Anguis fragilis* containing the most advanced embryos which we obtained, with a large amount of yolk not yet absorbed, the ovaries were very much reduced, and strongly pigmented. They were scarcely thicker than a match, and only a few small ova were visible in them. Corpora lutea could not be distinguished by ordinary vision, but the sections under the microscope, as seen in those exhibited, show that

the greater part of the ovary consists of these structures, no less than three of which may be seen in a single section, while ova containing yolk are entirely absent. The presence of embryos in the oviducts is associated with not only the persistence of these corpora lutea, but with the almost complete suppression of the development of ova. Ova in the earliest stages of growth are only present in one or two limited regions of the periphery of the sections. Where the wall of the corpus luteum is united with the external tissue of the ovary, the latter is covered with a conspicuous and peculiar layer of cells having the appearance of columnar epithelium, in strong contrast with the germinal epithelium on the surface of the rest of the ovary. Apparently this columnar structure is the active condition of the germinal epithelium which will later give rise to new ova.

There has been much controversy concerning the parts played by follicular epithelial cells and those of the theca interna. The sections of the early stages of the corpora lutea in reptiles studied by us show very clearly the distinction between these two sets of cells, the theca cells being external to the follicular. In later stages the former penetrate among the latter, but the two kinds can still be distinguished by the size of their nuclei. In the oviparous form *Lacerta* the discharged follicles immediately after ovulation are very similar to those of the viviparous *Anguis* and *Zootica*. In both cases the ova develop large yolks and attain a comparatively large size, while the earlier stages have remarkably thick follicular epithelium. In the oviparous form there is no indication of progressive development in the discharged follicles, reduction and absorption commence immediately after ovulation. In the intermediate stages we noticed in the interior of the follicle undergoing reduction very large cells which appear to be phagocytic in action, and further investigations are being carried out on the origin and functions of these cells. In two specimens of *Lacerta viridis* ovulation appeared to be induced by anterior pituitary injection, because each of them shed fully developed eggs the day after the injection.

So far as they go, these experiments support the view that the corpora lutea of the mammal are to be regarded as the effect of gestation. The question as to how this conclusion can be harmonized with the action of these structures as organs of internal secretion must be considered in the future.

Calcium tolerance in cats. By JOHN L. D'SILVA.
(*Physiological Laboratory, King's College, London.*)

Intravenous injection of calcium chloride dissolved in Ringer's solution (18 mg. Ca/1 c.c.) into chloralosed cats resulted in a rise in serum calcium, the extent of the increase differing from one animal to another.

Experiments on four cats are now reported as these animals showed a remarkable tolerance for large quantities of calcium in contrast to certain others for which calcium chloride was highly toxic. The fatal effect could not be ascribed to differences in the rates of injection, which were constant at about 10 sec. per 1 c.c.

Calcium was estimated by the Trevan and Bainbridge [1926] method, as modified by Patterson [1930].

Description of animal	Ca injected	Initial serum calcium	Calcium levels during experiment
11. (M.) 2 kg. 19. x. 32	416 mg. Ca in small doses in 2·5 hr.	10·4	26·3 mg. after 174 mg. Ca
13. (Preg.) 3·2 kg. 31. x. 32	110 mg. Ca in dose	9·0	13·6 mg. 5 min. after injection
9. (F.) 2 kg. 13. x. 32	422 mg. Ca in small doses in 5 hr.	10·3	19·0 mg. after 74 mg. Ca 43·1 mg. after 239 mg. Ca 53·5 mg. after 422 mg. Ca
80. (M.) 2·5 kg. 23. vi. 33	288 mg. Ca in small doses	7·9	37·6 mg. after 288 mg. Ca

It is seen from the table that the tolerance is not related to the initial serum calcium, but appears to be due to a great capacity of the tissues to assimilate calcium. The tolerance of such a high serum calcium as that shown by Cats 9 and 80 suggests that the calcium is in an inactive form in the blood; otherwise calcium rigor of the heart would be produced.

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The influence of adrenaline on the blood potassium. By JOHN L. D'SILVA. (*Physiological Laboratory, King's College, London.*)

Intravenous injection of 0·5 c.c. 1/10,000 adrenaline into chloralosed cats resulted in a large (up to 65 p.c.) increase in the concentration of the serum potassium as estimated by the method of Kramer and Tisdall [1921]. Within 15 min. the serum potassium value returned to its original level, or just below.

The injection was accompanied by an increase in the whole blood potassium, this probably being due in part to the entry of red corpuscles into the circulation, though hæmatocrit determinations before and during adrenaline action were practically identical.

The increase in serum potassium appears not to be due to increased permeability of the red cells, as the action of adrenaline on blood *in vitro* does not produce a similar result. The serum potassium of blood drawn under oil into paraffined vessels and then treated with adrenaline may show a small increase (up to 1 mg.), but this is almost certainly due to hæmolysis. The entry of potassium into the blood stream from the tissues appears to be highly probable.

The investigation is being continued and extended.

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PROCEEDINGS

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A sensitive physical test for adrenaline. By J. H. GADDUM and H. SCHILD.

Paget [1930] and Barker, Eastland and Evers [1932] have noticed that adrenaline, in the presence of alkali and various other reagents, becomes fluorescent. Adrenaline, oxygen, and alkali are, however, the only reagents which are essential for the effect. This reaction can be used as the basis of a test for adrenaline which is more sensitive than other known chemical tests, and in certain respects unexpectedly specific.

In our experiments the solutions were contained in small quartz tubes, with a capacity of about 1 c.c., and illuminated horizontally by light, from a mercury vapour lamp, filtered through a glass which absorbed practically all visible rays. The intensity of the fluorescence in two similar tubes, containing different solutions, was compared by observing them directly from above.

In faintly acid solutions adrenaline showed no fluorescence, but if 0·1 c.c. of 5 *N* NaOH was added to 1 c.c. of adrenaline solution, a bright apple-green fluorescence appeared, which lasted for about a minute with low concentrations and for several minutes with higher concentrations. The fluorescence lasted longer when oxygen was partially excluded, but did not appear at all in the complete abscence of oxygen. A green colour definitely different from the pale blue of the distilled water or diluted NaHO was seen when the concentration of adrenaline was only 10^{-6}. With double the concentration of adrenaline the effect was obviously greater.

Various substances chemically related to adrenaline were also tested. None of these gave a colour nearly so bright as that given by adrenaline in the same dilution, but in certain cases a fainter green fluorescence did develop on the addition of alkali. The intensity of this was estimated by comparing solutions of different strengths with adrenaline solutions, until the intensities of light in the two tubes were about equal. The comparison was made in every case 20 sec. after the addition of alkali. In the following list of substances tested, the figures give the intensity of

the fluorescence, estimated in this way, as a percentage of that due to adrenaline: "Dopa," 3 p.c.; noradrenaline, 2 p.c.; epinine, < 2 p.c.; catechol, 0·1 p.c.; tyramine and ephedrine, nil. The duration of the fluorescence due to these different substances was inversely related to its intensity.

When we attempted to apply this test to blood serum, we found that the fluorescence due to low concentrations of added adrenaline was obscured by blue fluorescence due to other substances. We have not been able to discover a method of completely removing these other substances. Deproteinization by means of trichloracetic acid or metaphosphoric acid was found to remove added adrenaline, when this was present in low concentrations. It is possible, however, that this test might be used to demonstrate the liberation of adrenaline from tissues into other simpler liquids such as Ringer's solution.

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Nomenclature of fibres in the autonomic system and their effects. By H. H. Dale.

Evidence has been rapidly accumulating in favour of the view that the effects of nervous impulses in the postganglionic fibres of the autonomic system are chemically transmitted, by substances liberated in relation to their endings, and directly acting on the effector cells. The conception was first put forward by Elliott, in 1904, with regard to sympathetic nerve endings and adrenaline. Really convincing experimental evidence in its favour, for both parasympathetic and sympathetic nerve impulses, was first obtained by O. Loewi, in 1921, and in recent years it has rapidly multiplied. My own earlier observations [1914] on acetylcholine had indicated a substance of this kind as a likely candidate for the function of transmitting the effects of impulses in peripheral parasympathetic fibres; and the evidence in recent years has all tended to increase the probability that this choline ester is the actual parasympathetic transmitter. We have as yet, however, no direct chemical evidence as to the nature of the peripheral transmitter for the other main division of the autonomic system (Cannon's "sympathin"), though physiological evidence indicates a close relation, at least, to adrenaline. Moreover, the correspondence between anatomical origin and functional chemistry is not exact. There are several

examples—*e.g.* the production of sweat secretion, sympathetic vasodilatation and paradoxical contracture in the dog's lips—of effects produced by fibres which are anatomically sympathetic, but apparently transmitted by something like acetylcholine, rather than by something like adrenaline. A further complication arises from the new fact, communicated to-day by Feldberg and Gaddum, that chemical transmission occurs at synapses in a sympathetic ganglion, and that the transmitter, even though the preganglionic fibres are anatomically sympathetic, is a substance indistinguishable from that which transmits the effects of postganglionic parasympathetic impulses. The same point arises in the account by Feldberg, Minz and Tsudzimura of chemical transmission from preganglionic sympathetic fibres to cells of the suprarenal medulla. Both these observations bring into the physiological picture the other aspect of the action of acetylcholine, which I called "nicotine-like."

To avoid elaborate periphrasis, and to promote clear ideas, we seem to need words which will briefly indicate action by two kinds of chemical transmission, due in the one case to some substance like adrenaline, in the other case to a substance like acetylcholine, so that we may distinguish between chemical function and anatomical origin. I suggest the words "adrenergic" and "cholinergic," respectively, for use in this sense. We can then say that postganglionic parasympathetic fibres are predominantly, and perhaps entirely, "cholinergic," and that postganglionic sympathetic fibres are predominantly, though not entirely, "adrenergic," while some, and probably all, of the preganglionic fibres of the whole autonomic system are "cholinergic." I think such a usage would assist clear thinking, without committing us to precise chemical identifications, which may be long in coming.

Acclimatization (P) of animals to 0·3 p.c. carbon monoxide in the inspired air. By J. Argyll Campbell.

These observations form the continuation of a research to determine the ill-effects, if any, of breathing CO as present in garages, etc., for prolonged periods.

Some young mice and rabbits exposed to CO in the air in gradually increasing concentrations can maintain body growth when inhaling as much as 0·3 p.c. Indeed in mice there is evidence of increased growth compared with the controls, the body weight being increased and the heart revealing hypertrophy. The latter was noted also in some previous experiments. The improved body growth cannot be regarded as a proof

of acclimatization, because the mice exposed to the poisonous gas were not fertile, whereas the controls, similarly treated as regards food, surroundings, etc., were fertile.

Fenn and Cobb [1932] observed that *in vitro* frog's skeletal muscle consumed CO—1 c.mm. per g. per min. at 22° C.—when the gas was present in 80 p.c. concentration. In the present research there was no evidence that the whole animal consumes such a material quantity of CO when breathing 0·3 p.c. for long periods. If, however, we assume that the consumption of the poison by muscle is proportional to the concentration—which perhaps seems doubtful for living tissue—then from Fenn's figures we would expect a rabbit of 2 kg. (muscle 40 p.c.) to consume only about 14 c.c. in 24 hours when exposed to 0·3 p.c. There was no evidence of this quantity being used, but it is too small a quantity to be estimated very accurately (Haldane combustion chamber, large apparatus) under the conditions of the experiment, the respiratory chamber for the animal being about 150 litres gas capacity. In these experiments it was necessary to starve the animal for 2 days to exclude formation of other combustible gases, *e.g.* hydrogen, etc., from the food in the intestines.

The tension of CO in rabbit's tissues was also estimated by establishing gas depots in the abdominal cavity and under the skin. When the animal had breathed 0·3 p.c. CO for prolonged periods, the tension of CO in the tissues was about 0·15 p.c., the analysis of the gas being conducted in the large Haldane analyser. These results give some support to previous work [Campbell, 1929] when the Hartridge spectroscope was used for estimation of CO; in the latter case the tension found was somewhat lower.

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The chemical transmitter at synapses in a sympathetic ganglion. By W. Feldberg and J. H. Gaddum.

Kibjakow [1933] perfused the superior cervical ganglion of a cat with Locke-Ringer solution, and found that stimulation of the cervical sympathetic nerve caused the appearance in the outflowing fluid of a substance which, when injected into the fluid flowing to another ganglion, caused a contraction of the nictitating membrane. Chang and Gaddum [1933] found that extracts of the sympathetic chain of a horse contained

comparatively large amounts of a substance identified pharmacologically as acetylcholine, and suggested that the active principle detected by Kibjakow might also be acetylcholine. This view was strengthened by the discovery that stimulation of (preganglionic) sympathetic fibres to the suprarenal medulla caused the liberation of a choline ester [Feldberg, Minz and Tsudzimura, 1933].

In order to test this theory we have perfused the superior cervical ganglia of cats with salt solutions, and applied various pharmacological tests to the outflowing fluid. In the absence of eserine (physostigmine) no activity was detected, but when eserine was added to the perfusion fluid, in order to inhibit the destruction of acetylcholine, it was found that fluid collected during stimulation of the preganglionic nerve had the following properties:

(1) Like acetylcholine, it had no action on a piece of leech muscle untreated with eserine, but it produced a contraction when the preparation had previously been sensitized to acetylcholine by prolonged immersion in eserine. ("Nicotine action.")

(2) It caused inhibition of a frog's heart isolated by Straub's method. This effect was abolished by atropine. ("Muscarine action.")

(3) It caused a fall of blood-pressure in a cat. This effect was also abolished by atropine. ("Muscarine action.")

The solutions were compared with acetylcholine and a quantitative estimate of the concentration was obtained. It was found that when the same solution was tested simultaneously on the frog's heart, and on the leech or the cat's blood-pressure, these estimates agreed quantitatively with one another. We regard these results as strong confirmation of the view that these solutions contained acetylcholine, in a concentration of between 20 and 70 γ per litre. Fluid collected in the absence of stimulation and tested in the same concentrations had none of these actions.

Since choline esters are active stimulants of sympathetic ganglia, it seems probable that the liberation of such a substance forms the normal mechanism by which the effects of preganglionic impulses pass synapses in the ganglion. Our results support the theory that this substance is acetylcholine.

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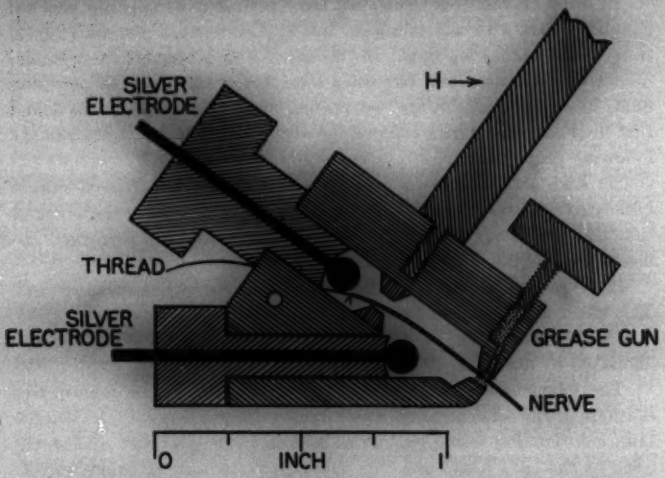
A convenient form of fluid electrode. By L. W. COLLISON. (Introduced by H. H. DALE.)

The fluid electrode figured is a modification of that described by Brown and Garry [1932], and has the following features which have been found to give additional convenience in construction and use.



1. The instrument is wedge-shaped in section, and this enables the vulcanite plugs, used in filling the chamber with blood and sealing it, to carry the silver electrodes, for which additional openings are not required. It also enables the instrument to be placed in position in a deep fossa, and to take a relatively small length of dissected nerve.

2. The brass holding-rod (*H*) enables the electrode to be clamped in position.

3. The vulcanite screw functioning as a "grease-gun" greatly facilitates the sealing with vaseline of the hole through which the nerve is drawn into the chamber. The sealing and filling of the electrode are thus normally and easily effected after the apparatus has been fixed in position.

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The mechanism of the nervous discharge of adrenaline.

By W. Feldberg, B. Minz and H. Tsudzimura. (Introduced by H. H. Dale.)

Evidence for the peripheral chemical transmission of the effects of autonomic nerves, both sympathetic and parasympathetic, has accumulated in recent years. The question presented itself whether the impulses in (preganglionic) splanchnic fibres, which cause output of adrenaline from the cells of the suprarenal medulla, produce this effect also by a chemical transmission, and, if so, whether the transmitter is of the sympathetic (adrenaline) type, or of the parasympathetic (acetylcholine) type. We have been able to find evidence that a chemical transmission occurs in this case, and that the transmitting agent, directly affecting the medullary cells, has all the properties of acetylcholine, so far as these can be tested.

1. The body wall of the leech treated with dilute eserine is an extremely sensitive and specific reagent for acetylcholine, contracting in response to dilutions as high as 1γ per litre [Führer, Minz]. It is, on the other hand, quite insensitive to adrenaline, and practically so to the constituents of normal mammalian blood. If eserine is injected into a dog or cat and the splanchnic nerve is stimulated, the blood leaving the suprarenal vein acquires during the stimulation, but only then, a stimulant action on the leech preparation, corresponding to that of acetylcholine in dilutions of 10–100γ per litre. The active substance disappears rather quickly from the shed blood, but persists if additional eserine is mixed with it. It has, therefore, the properties of an unstable choline ester.

2. In an eviscerated cat under eserine, the adrenaline rise of blood-pressure caused by stimulation of the splanchnic nerve is preceded by a fall, which a small dose of atropine prevents. If an excess of adrenaline has already been put into circulation, by injecting 2–3 mg. before the splanchnic is stimulated, the atropine-sensitive fall is the only effect of the stimulation.

3. Acetylcholine-like effects, produced by splanchnic stimulation in an eviscerated cat under eserine, can be demonstrated on a distant organ. If the effect of adrenaline on the submaxillary gland is further excluded by injecting 2–3 mg. of ergotoxine, stimulation of the splanchnic nerve causes secretion of saliva, which atropine again abolishes.

4. In an eviscerated cat under atropine, eserine increases the output of adrenaline in response to splanchnic stimulation, as measured by the

rise of blood-pressure. This indicates that the output of adrenaline is due to the liberation of a substance which eserine protects—i.e. a choline ester.

5. Acetylcholine itself is a very potent stimulant of adrenaline output from the suprarenal medulla, when it is injected into the arterial stream on its way to the gland. The action is due mainly to the "nicotine" aspect of its action, being almost abolished by large doses of nicotine, which, similarly, almost abolish the adrenaline output in response to splanchnic stimulation. In both cases nicotine often leaves a small residue of effect, always when eserine has also been given, and this residue, due to the "muscarine" action of acetylcholine, is abolished by atropine.

The chemical transmitter of splanchnic action on the suprarenal medulla has in all respects the properties of an unstable choline ester with both "nicotine" and "muscarine" actions, of which the former is in this case predominant.

The chemical transmitter of effects of the gastric vagus.

By H. H. Dale and W. Feldberg.

The fact that the motor effects of the vagi and the pelvic nerves on the muscular walls of the alimentary canal are so resistant to the action of atropine has been an obstacle to the general acceptance of acetylcholine, or any choline ester, as the probable chemical transmitter of these parasympathetic effects. It seemed important, therefore, to attempt to obtain the chemical transmitter of such an effect, so as to examine its properties separately. This has been done in the case of vagus action on the stomach.

In dogs under chloralose the venous flow into the portal vein was restricted to blood from the stomach, by ligature and excision of other organs. Arrangements were made to collect samples of the portal (gastric) blood. The vagi were exposed in the lower thorax and dissected from the œsophagus for stimulation. A large dose of eserine (physostigmine) (1–2 mg. per kg.) and a smaller one (0·5 mg.) of atropine were given intravenously, and the blood was rendered incoagulable with heparin or a substitute. Samples of portal blood were collected before stimulation of the vagus, and during stimulation adequate to produce powerful contraction of the stomach wall. Eserine was added to the samples as collected to the extent of 1 in 50,000. They were tested on:

(1) the leech body wall, sensitized to acetylcholine with eserine (nicotine action);

(2) the blood-pressure of a cat under chloralose (muscarine action).

In all cases the effects produced by one blood sample in these two directions could be matched against the same concentration of acetylcholine. When a large eserine injection had been given, the control blood showed activity which matched with about 20γ of acetylcholine (A.C.) per litre. Vagus stimulation then raised the activity about 2½ times, to match with 50γ A.C. per litre. With a smaller dose of eserine the activity of the control blood was below the limit of measurement (not more than 5γ A.C. per litre), while that of the blood taken during stimulation matched with about 20γ A.C. per litre.

The effects on the cat's blood-pressure were always abolished by 0·1 mg. of atropine, and the leech untreated with eserine gave no specific reaction, with dilutions producing maximal contractions in the sensitized preparation. From blood samples collected without added eserine the activity rapidly disappeared.

Apparently the relatively atropine-resistant effect of the vagus on the stomach is transmitted by a substance having all the properties of an unstable, atropine-sensitive choline ester, indistinguishable from those of acetylcholine, so far as they can be tested in the venous blood collected during vagus stimulation.

Human electro-cardiograms recorded with the cathode ray oscillograph. By PIERRE RIJLANT. (Introduced by C. Lovatt Evans.) (*Institut Solvay. Physiology—University of Brussels.*)

In previous communications [1931] we have published human electro-cardiograms taken with a cathode ray oscillograph. These records have a general shape different from that of string galvanometer records. Fast and slow waves, not noticed in galvanometer records, exist in the oscillograph records. Similar waves have been noticed in oscillograph records by Reid [1932] and by Groedel [1933]. Matthews [1933] has recorded human electro-cardiograms with a cathode ray oscillograph. His records are not, at first sight, different from string galvanometer records and show neither fast nor slow extra-waves. Matthews suggests that the waves of the oscillograph records might be due to the amplifier rather than to any activity of the heart. As Matthews overlooked the publication of the exact details of the amplifiers we used [1932], he suggested that the distortion might occur in a capacity resistance distorting circuit of short time constant. The exact details of the amplifiers [1932] and the calibration curves [1933] show that there cannot be any noticeable distortion of

the cardiac waves in our records. To introduce distortion the time constant of our amplifier has to be reduced to one-hundredth of its actual value.

Though for these reasons, complete certitude exists as to the real shape of the human electro-cardiogram as recorded with a cathode oscillograph, we have tested out our previous findings, by the use of a battery coupled amplifier, as distortionless as at present possible [1933], giving the same range of amplification as the amplifiers previously used (four valves).

Cathode ray oscillograms of the human heart taken by this method have exactly the same general shape as the curves recorded with a resistance capacity coupled amplifier of long time constant. The records clearly show the presence of fast waves originating in the sinus and auricles (P 1–P 5), in the His-bundle (*h* 3) and of the slow waves (*h*, S 2, T 2) which Matthews did not see in his records. Inadequate amplification (three valves) seems to be the reason why Matthews's records do not clearly show the fast and slow waves of our records. Record C of Matthews shows clearly, when enlarged, a positive S 2 wave, corresponding to a 100 microvolt potential change (the average value of the S 2 wave in our records).

The site of origin of the waves of the cathode ray oscillogram of the human heart has been traced by using thoracic leads and by comparing the records obtained under these circumstances with the records obtained with the classical leads D I, D II, and D III. Comparison of records of the human electrogram with those of the dog's heart do not show any striking difference as the dog's records show exactly the same slow and fast waves as the human record. In the dog the heart potentials have been led off by thoracic leads and by electrodes directly put on the exposed heart. Both records show the same waves.

The fast and slow waves of the human electro-cardiogram recorded with the cathode ray oscillograph are due to the activity of the heart and not to a modification of the heart's action-potential by conduction through the surrounding tissues.

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Glycolytic formation of blood lactate. By C. Lovatt Evans, Fong-Yen Hsu[1] and Takao Kosaka[2]. (*University College, London.*)

It has been shown [Evans *et al.*, 1933] that the heart uses much more of lactate than of sugar from the blood (confirming McGinty); further, that the balance of continual formation of lactic acid by glycolysis in the blood, and its continual removal by the heart (or other tissue) might account for the phenomenon described in the heart-lung preparation by Anrep and Cannan [1923], and in the limb-lung by Eggleton and Evans [1930]. The margin of lactate formation was, however, apparently small, and this is particularly so if the argument be extended to the intact body in explanation of the normal resting blood lactate [Cook and Hurst, 1933].

We have now studied the rate of lactate formation in defibrinated blood perfused through ventilated lungs, and find that glycolysis is two or three times as rapid when blood is so circulated as when oxygenated by circulation *in vitro*. Removal of one lung does not seem to lower the rate of glycolysis if the blood flow through the remaining lung is the same as that previously through both. When the blood which has been passed through the lungs and that which has circulated through the oxygenator are interchanged after a period, the same difference is seen, *i.e.* that now passing through the lungs is the more rapidly glycolysed. In the lung about 80 p.c. of the glucose lost is converted into lactic acid; in the oxygenator the conversion is rather irregular, which may be associated with hæmolysis.

The following is an abstract of an experiment:

Hours from start	Lung circuit mg./100 c.c. blood				Oxygenator circuit mg./100 c.c. blood			
	Glucose		Lactic acid		Glucose		Lactic acid	
0·00	126	30	71	25	142	17	65	17
1·00	96		96		125		82	
2·00	67	29	129	33	106	19	104	22
2·01	Circuits interchanged							
2·18	85	27	126	15	78	14	129	5
3·18	58		141		64		134	
4·18	25	33	156	15	57	7	148	14

[1] China Foundation Fellow.
[2] Rockefeller Foundation Fellow.

In one experiment the lactate in the lung circuit rose at a rate of about 25 mg./100 c.c./hr. until all the blood sugar had disappeared and then fell 5 mg./100 c.c. in the next hour, thus indicating the origin of the lactic acid.

We suggest that the accelerated glycolysis in the lungs gives enough lactic acid to account fully for the phenomena mentioned, and probably for the formation of the resting blood lactate in the intact body. Further investigation is in progress.

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Duality of the mechanisms of tonus and contraction in striated muscle. By Pierre Rijlant. (Introduced by C. Lovatt Evans.)
(*Institut Solvay. Physiology—University of Brussels.*)

Two types of action currents can be led off by needle electrodes from a normally innervated striated muscle: fast waves of high potential, corresponding to contraction; much slower and smaller waves corresponding to tonic activity. The average amplitude of the "tonic" waves is one-third of the amplitude of the "contraction" waves in single motor units. The duration of the tonic waves is twice that of the contraction waves. The average frequencies of the contraction waves (30 per sec.) are higher than those of the tonic waves (10 per sec.), but the inferior limit of both is about the same (5 waves per sec. in mammals).

During the hypnotic sleep in mammals, the contraction waves disappear completely, except for the contraction waves of respiratory muscles, while the tonic waves remain unchanged. Deep hypnotic sleep abolishes the tonic reactions as well as contraction. When given in small amounts, curare completely abolishes the tonic waves while the contraction waves are maintained temporarily.

Two types of action currents can be led off from the central end of a motor nerve. They show the same characteristics as the two types of waves led off from the striated muscle which are related to tonus and contraction.

From human striated muscles (triceps, quadriceps) the same action currents can be led off. The fast contraction waves are frequently

built up by four to ten very fast waves slightly asynchronous. Some outbursts of tonic waves are related to the respiratory cycle. Other rhythmic outbursts of tonic waves, not related to the respiratory cycle, of an average duration of one second, are probably related to the Traube Hering oscillations of blood-pressure.

In the male amphibian, during the mating season, the tonic activities of the striated muscles of the forearm are accompanied by electrical waves of very long duration (100–200σ). These waves originate in the same fibres which normally are responsible for the tonic waves (10–20σ). The action currents of the motor nerves of the forearm are not different during the mating season from what they are under normal circumstances.

The change in duration of the tonic waves is only due to the change in the reaction of the striated muscle. The long duration of the tonic waves during the mating season suggests a humoral transmission at the neuro-muscular junction.

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The sensitization of leech muscle to barium by eserine.

By A. Vartiainen. (Introduced by H. H. Dale.)

Führer [1918] found that eserine caused a very remarkable sensitization of the dorsal muscle of a leech to acetylcholine, so that after eserine it reacted to a millionth of the concentration which had been necessary to cause a contraction before eserine. He suggested the theory that this sensitization was due to the inhibition of choline esterase, and recent work has confirmed this view.

Führer also found that eserine sensitized the leech to barium. This observation is of importance, since it seems to weaken arguments based on the assumption that any effect which is increased by eserine must necessarily be due to a choline ester.

Führer's experiments with barium and eserine have therefore been repeated. The fact that eserine has some sensitizing action on the response of barium was confirmed, but this sensitization was not so striking as might be supposed from Führer's papers, and was not comparable with that to acetylcholine. When repeated doses of barium were applied at constant time intervals to an isolated piece of leech muscle, left in contact with the muscle for 3 min. and then washed out, successive doses

produced increasing contractions, so that a certain amount of apparent sensitization occurred even in the absence of eserine. After some time, however, this increase in sensitivity ceased, and successive small doses produced a constant effect. If eserine was now added to the bath it caused some increase in the response to barium, but only to such an extent that the response was now equal to that produced by not more than twice the concentration of barium, before eserine.

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PROCEEDINGS

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December 16, 1933.

Action potentials from the superior cervical ganglion.

By J. C. Eccles.

Electrodes have been applied to the cat's cervical sympathetic trunk 4 or 5 cm. below the superior cervical ganglion and single induction shocks have been used as stimuli. By means of non-polarizable leads, action potentials so set up in the superior cervical ganglion have been led to an amplifier connected to a Matthews' oscillograph, the earthed lead being on the ganglion and the grid lead on the crushed end of the post-ganglionic trunk. The blood supply of the ganglion and of the cervical sympathetic has not been interfered with, but the vagus and the ganglion nodosum have been removed.

A complex negative wave appears in the ganglion 5–7 σ after stimulation of the cervical sympathetic, attaining at 12–17σ after the stimulus a maximum which may be as high as 0·5 mv. There are three components in this wave, the summit being formed by the second component, while the third appears as a small hump late on the descending phase. Finally a fourth negative wave becomes first recognizable 30–40σ after the stimulus, reaches a low maximum at 45–60σ and is still usually present at 70σ. As the strength of the stimulus is gradually weakened, the fourth wave may be the first to disappear, and then the third, second and first waves may disappear in turn. However, the thresholds characteristic of each of these waves are not very sharply differentiated, *e.g.* the fourth wave has been present with strengths of stimuli which were submaximal for the first and second waves.

A study of the pre-ganglionic action potentials shows two waves, one conducting at 20–25 m. per sec., the other at about 15 m. per sec. The first pre-ganglionic impulses therefore arrive at the ganglion 2–2·5σ after the stimulus, so there is a latent period in the ganglion of at least 3σ before the ganglionic action potential appears.

When a second pre-ganglionic stimulus is applied at an interval as short as 3·7σ after a previous stimulus, it gives rise to an action potential

in the ganglion. The latent period of the onset of this potential may be 1σ shorter than normal, and the first potential wave rises much more steeply than normal. It is of course superposed on the potential wave set up by the first stimulus. Since the pre-ganglionic conduction time for the volley set up by the second stimulus is increased by about $1{\cdot}5\sigma$, this pre-ganglionic volley must arrive at the ganglion only 1σ before it sets up a ganglionic action potential, *i.e.* the latency in the ganglion has been considerably shortened. The second ganglionic wave is also set up by a stimulus applied $3{\cdot}7\sigma$ after the first, but its latent period appears increased by an amount equal to the lengthening of the pre-ganglionic conduction time. The third wave is absent at $3{\cdot}7\sigma$ interval, but present when the interval was $5{\cdot}7\sigma$, and the fourth wave only appears after stimuli at 12–15σ intervals, its maximum being attained at intervals greater than 32σ. The first three waves appear normal in size after stimuli applied at intervals of 12σ or longer. The threshold and refractory period for the setting up of pre-ganglionic nerve impulses are identical with the values obtained for the ganglionic action potentials.

The various component waves in the ganglionic action potential could be ascribed to pre-ganglionic fibres with conduction times corresponding with the delay in the appearance of the respective waves [cf. Bishop and Heinbecker, 1932], *e.g.* the fourth wave could be due to the *C* fibres which conduct at 1–2 m. per sec.; but the waves appear, though with a diminished latency, even when the pre-ganglionic trunk is stimulated just proximal to the ganglion, so it seems that the greater part of the delay in the appearance of the second, third and fourth waves must be due to a delay in the ganglion corresponding with that found for the first wave.

When the blood supply to the ganglion is cut off, the latent period of the ganglionic action potential is increased to as much as 15σ, the summit being reached as late as 27σ, while a second stimulus even as late as 12σ after the first fails to set up any ganglionic action potential, though it gives rise to a full-sized pre-ganglionic wave. Bishop and Heinbecker [1932] state that there is an absolutely refractory period of 20σ and a synaptic delay of 10σ for conduction through the superior cervical ganglion, whereas Brown [1933] finds that both refractory period and synaptic delay are less than 3σ. This discrepancy is no doubt due to the fact that Bishop and Heinbecker worked on excised ganglia.

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The effects of eserine and atropine on the vagal slowing of the heart. By J. C. Eccles.

In a previous communication [Brown, Eccles and Hoff, 1932] the inhibitory effect of single vagal volleys on the heart rhythm was described, and the time course of this inhibition was depicted by plotting curves with lengthenings of cycles as ordinates and the corresponding intervals after the vagal stimuli as abscissæ. An initial maximum inhibition is always attained about 0·5 sec. after the vagal stimulus, and is followed by a rapid decline, the inhibitory effect falling to half its maximum in 0·4–0·8 sec. This rapid inhibitory wave is followed by a slower inhibitory wave first perceptible 0·6–1·5 sec. after the vagal stimulus, the maximum being reached 2–2·5 sec. after the stimulus. When the first inhibitory wave is high and long-lasting it obscures the onset and even the summit of this second inhibitory wave, which may appear merely as a hump or flattening on the descending part of the first wave. At the summit of the first wave there may be even more than a doubling of the duration of the cardiac cycle, but the maximum of the second wave is never more than a 10 p.c. lengthening.

As the stimulation of the vagus is progressively made more and more submaximal the height of the first wave declines much more rapidly than the second, and with small vagal effects the second wave may be higher than the first. The inhibitory curve of the left vagus is always identical with a submaximal inhibitory curve of the right vagus. When a second vagal volley is set up (either in the same or other vagus) at any interval after the first, its first wave always adds directly on to the inhibitory curve produced by the first volley, but its second wave is always largely occluded by the second wave set up by the first volley. Tetanic excitation of the nervi accelerantes always diminishes the second wave and may cause it to disappear.

The following possible explanations of the double inhibitory waves have been excluded:

(1) The trough between the two inhibitory waves being due to a transient displacement of the pacemaker to another part of the sino-auricular node.

(2) The trough between the inhibitory waves being due to a transient accelerator action from accelerator fibres in the vagus.

(3) The second wave being due to after-discharge from the post-ganglionic neurones of the vagus.

It seems therefore that the double inhibitory wave is an expression of a double inhibitory effect produced by a single volley of impulses arriving at the terminals of the post-ganglionic fibres of the vagus.

Eserine and atropine have been used to obtain further evidence on the nature of these two inhibitory waves. Eserine given intravenously in doses of 25γ to 2–3 kg. cats increases the height of the first wave and approximately halves the rate of its decline. The height of the second wave is similarly affected but to a relatively smaller extent. The maximum effect is attained in 3 min. after the injection and it has completely passed off in 2–3 hours. Doses of eserine several times larger so prolong the first wave that it completely overlaps the second. Atropine in very small doses, *e.g.* 5γ, lowers the inhibitory curve so that it becomes identical with the curve produced by submaximal stimulation, *i.e.* the height of the first wave is reduced relatively much more than the height of the second wave. This indicates that the inhibitory processes responsible for the two waves are similarly affected by atropine.

Thus the effects of eserine and atropine indicate that both inhibitory waves are due to the same inhibitory substance, presumably acetylcholine, and the effect of eserine indicates that all phases of the decline of the inhibitory curve are due to the enzymatic hydrolysis of acetylcholine. The concentration of eserine which halves the rate of this hydrolysis corresponds approximately with that found by Matthes [1930] in *in vitro* experiments on the hydrolysis of acetyl-choline.

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Blood gas figures in a case of Fallot's tetralogy. By M. KREMER.
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J. R., a Welsh miner aged 35, had been noticing increasing blueness during the last two years with marked shortness of breath. There were occasional attacks of giddiness with faintness but no loss of consciousness.

Examination showed slight enlargement of the heart to the left, and this was confirmed by X-ray which showed a "sabot shaped" heart. There was a systolic thrill and murmur in the third left interspace about ½ in. from the sternal edge. Very marked cyanosis and clubbing of fingers and toes. A diagnosis of Fallot's tetralogy was made.

By means of arterial puncture an attempt was made to calculate the arterio venous shunt. Blood was taken from the femoral artery and its

oxygen content and capacity estimated by means of the Haldane blood gas analysis apparatus.

Duplicate estimations gave the following results: oxygen content = 17·75 c.c. p.c.; oxygen capacity = 34·75 c.c. p.c.; saturation, p.c. = 51.

Further estimations were done after the patient had inhaled pure oxygen for ½ hour through a nasal catheter. Oxygen content rose to 22·1 c.c. p.c. and percentage saturation to 64 p.c.

The oxygen capacity was also determined by colorimetric and gravimetric estimations of hæmoglobin. The Hb by the Haldane standard was 140 p.c. (= 20 g. Hb = 27 c.c. O_2 p.c.). Gravimetric estimation was 26 g. per 100 c.c. = 34·5 c.c. O_2 p.c. It will be seen that the gasometric estimation agrees closely with the gravimetric but not with the colorimetric, which is probably less reliable at high concentrations of hæmoglobin.

From the above gasometric data the degree of arterio venous shunt may be calculated. Assuming that all the unsaturation is due to shunt, then using the formula of Segall [1928] let X = venous shunt as from right to left side of circulation in p.c. of total blood flow and $100 - X$ = p.c. amount of blood entering arterial system from pulmonary veins, then

$$\text{Oxygen in arterial blood} = \frac{(\text{venous oxygen})\,X}{100} + \frac{(\text{oxygen in pulmonary veins})\,(100 - X)}{100}.$$

Following Lundsgaard it may be assumed that the oxygen in pulmonary veins is 95 p.c. of oxygen capacity and that oxygen in mixed venous blood is 5 volumes p.c. less than arterial oxygen content. Substituting these figures we obtain

$$17{\cdot}75 = \frac{(12{\cdot}75)\,X}{100} + \frac{33}{100}(100 - X),$$

which gives a venous shunt of 75 p.c.

An alteration of ±1 c.c. in venous oxygen content makes a difference of ±5 p.c. in venous shunt.

From the oxygen inhalation experiment it appears, however, that the unsaturation of the arterial blood is partly due to incomplete aeration of the venous blood in the lungs. Using the data of the inhalation experiment the shunt is calculated to be 69 p.c.

This probably represents a true index of the state of affairs existing.

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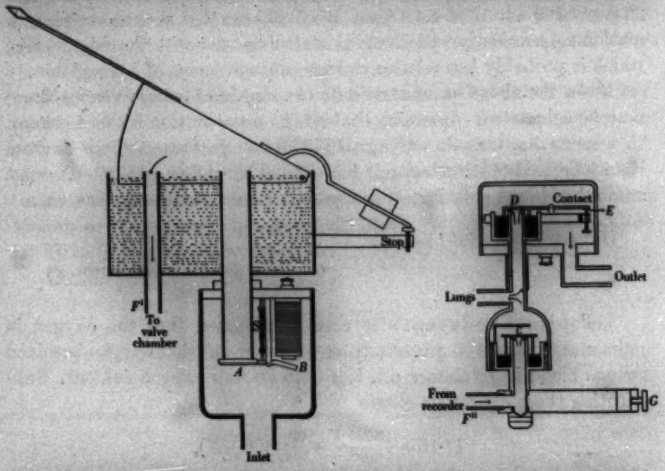
A quantitative graphic respiration recorder for small animals.

By SAMSON WRIGHT. (*Department of Physiology, Middlesex Hospital.*)

The apparatus consists essentially of a Krogh's spirometer with an outlet F^{I} which is connected at F^{II} with respiratory valves. The inspiratory and expiratory valves, *C* and *D*, are both of the hat type (*C* is made of mica and *D* of ebonite), and float on a pool of mercury. The inspiratory valve is prevented from being lifted excessively by a little shelf. During inspiration air is withdrawn from the spirometer and the writing point sinks. During expiration, when the valve *D* is raised, a contact is made



at *E*. This closes a circuit which is connected with the electromagnet *B*, and as a result the tambour valve *A* is opened. This enables air from the inlet chamber to refill the spirometer to its original position, over-swing being prevented by the stop. When expiration ends, contact at *E* is broken and the valve *A* (assisted by the action of the spring *S*) returns to the position shown in the diagram and shuts the spirometer off from the inlet. The apparatus can be readily calibrated so that it is accurately quantitative. If the effects of various gas mixtures are to be studied the inlet can be connected by means of corrugated rubber tubing with a Douglas bag. The spirometer should first be washed through several times with the experimental mixture. The resistance of the valves is exceedingly small (less than 2 cm. water) and the dead space is about

10 c.c. The expired air can be collected from the outlet. The record is unaffected by the movements of the animal other than respiratory. The spirometer has some inertia and the first five or six breaths recorded by it are less deep than the succeeding ones. When breathing is exceedingly shallow, as just prior to respiratory arrest, expiration may be too short to make the contact *E* or too brief to open the valve *A* for a sufficient time to refill the spirometer. This difficulty occurs rarely. I have had the apparatus in use for several months and found it otherwise quite reliable. It is made by C. F. Palmer, Ltd., and I am indebted to Mr E. Ellis for much technical assistance.

Muscle phosphorus compounds in adrenal insufficiency. By EINAR LUNDSGAARD and A. T. WILSON. (*Departments of Physiology, University of Copenhagen, and Middlesex Hospital.*)

Muscular weakness, a leading symptom of experimental adrenalectomy and of Addison's disease, has been related to a reported quantitative alteration in the phosphagen content of muscle. [Lang, 1931; Ochoa, 1932.]

In this research muscle specimens were obtained from cats between 5 and 8 days after bilateral adrenalectomy, when various gross symptoms were present. The animals were shot by a "humane killer" (or anæsthetized with "Nembutal"); a portion of muscle, cut out as rapidly as possible from rectus femoris, was dropped into liquid air.

For determination of the various phosphorus compounds, the methods of Lohmann and Jendrassik [1926], and Lohmann [1928] were used.

P_2O_5 in the form of inorganic phosphate, phosphagen, adenyl-pyrophosphate and hexose-phosphate was determined in five doubly adrenalectomized animals and also in five controls, unilaterally adrenalectomized, subjected to a second "dummy" operation, and given the same amount of food as was consumed by the completely adrenalectomized animals. The clinical and muscular condition of the controls was normal.

	Adrenalectomized		Controls	
(Average values in mg. P_2O_5 per g. of muscle.)				
Inorganic phosphate	56	159	65	155
Phosphagen	103		90	
Pyrophosphate	39		39	
Hexose-phosphate	46		60	

With one exception, the phosphagen range lay between 107 and 121 mg. per g. in the adrenalectomized animals.

The slight differences in the amount of these substances in the two groups may be due to differences in muscular activity at the time of death.

It seems improbable that the gross muscular weakness of the adrenalectomized animal can be related directly to the small alterations in muscle phosphorus compounds noted.

Typical protocol.

Cat 91. Male. 3·4 kg. Adrenalectomy in two stages. First symptoms on 3rd day after 2nd operation. Symptoms well marked on 7th day after which the animal was shot.

Blood urea: 215 mg. per 100 c.c. Blood non-protein nitrogen—118 mg. per 100 c.c.

Blood sugar: 42 mg. per 100 c.c. Fixed CO_2 of blood—34 c.c. CO_2 p.c.

Control Cat 82, unilaterally adrenalectomized and with second "dummy" operation. Clinical condition normal when shot on 7th day.

Blood urea: 31 mg. per 100 c.c.

N.P.N.: 30 mg. per 100 c.c.

Blood sugar: 150 mg. per 100 c.c. (emotional hyperglycæmia).

Fixed CO_2: 35 c.c. CO_2 p.c.

Figures given below are in mg. P_2O_5 per g. of muscle.

Adrenalectomized (No. 91)		Control (No. 82)
Inorganic phosphate	62	59
Phosphagen	110	91
Adenyl-pyrophosphate	40	35
Hexose-phosphate	50	65
Total P	262	250

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